IMMUNOMODULATION BY ISOMETAMIDIUM (SAMORIN®) ENHANCES PROPHYLAXIS AGAINST *TRYPANOSOMA CONGOLENSE* INFECTION

By

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DEDICATION

To my wife Lungowe, Milambo and Hibajene.
"May the next generation succeed where we have failed".
"In order to make progress, a turtle must be prepared to stick out its neck"
"Where there is a will, there is always a way"

DECLARATION

I hereby declare that, the work presented in this thesis was carried out entirely by myself unless where specifically stated in the text and acknowledgement

Kennedy Choongo

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ABBREVIATIONS

ATP	-	adenosine triphosphate
BCG	-	*Bacille Calmette-Guerin*
CO_2	-	carbon dioxide
CD	-	Cluster of differentiation
°C	-	degrees centigrade
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetracetate
ELISA	-	enzyme linked immunosorbent assay
FITC	-	Fluorescein isothiocyanate
γδ	-	gamma delta
Hrs	-	hours
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
IFN-γ	-	interferon gamma
IL-2	-	interleukin 2
IL-2R	-	interleukin 2 receptor
IL-10	-	interleukin 10
IL-12	-	interleukin 12
ISMM	-	Isometamidium (Samorin®)
kg	-	kilogram
LPS	-	lipopolysaccaride
M∅	-	macrophage
µg	-	microgram
µl	-	microliter
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
MEC	-	minimum effective concentration
ng	-	nanogram
nm	-	manometre
PCV	-	packed cell volume
%	-	percent
PBL	-	peripheral blood lymphocytes
PBMC	-	peripheral blood mononuclear cells
PBS	-	phosphate buffered saline
PBST	-	phosphate buffered saline tween 20
pg	-	picogram
PPD	-	purified protein derivative (tuberculin)
RBC	-	red blood cells
RNA	-	ribonucleic acid
TCR	-	T cell antigen receptor
TNF	-	Tumour necrotic factor
US$	-	United States dollar

VAT	-	variant antigen type
VSG	-	variable surface glycoprotein
VSSA	-	variant specific surface antigens
WBC	-	white blood cells

THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

(Regulation 3.5.13)

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Title of Thesis **Immunomodulation by Isometamidium (Samorin®) enhances prophylaxis against *Trypanosoma congolense* infection**

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The objective of this project was to investigate effects of isometamidium (ISMM, Samorin®) on immune responses to *Trypanosoma congolense* infection in order to understand mechanisms of prophylaxis. (1) the efficacy of ISMM on *T. congolense in vitro* and *in vivo* was examined. (2) effects on sheep peripheral blood mononuclear cell (PBMC) phenotypes, proliferation, and IFN-γ production were investigated. (3) effects on IL-12 and IFN-γ production by mice splenic cells were studied. (4) effects on sheep PBMC phenotypes following BCG vaccination in sheep were also investigated in order to establish whether effects of ISMM were specific for trypanosome antigens.

Three groups consisting of four sheep were used in the trypanosome study. One group was prophylactically treated with ISMM and one was used as a normal control group. Four and half months later, all sheep were infected with *T. congolense* plus a third group that was later treated with ISMM 14 days post infection. Two groups of three sheep were used in BCG experiments; one was first treated with ISMM and 14 days after treatment both groups were inoculated with BCG vaccine. The efficacy of ISMM on *T. congolense* was studied on cultures *in vitro* and in sheep and mice *in vivo*. IL-12 and IFN-γ production by mice splenic cells was investigated at different points after ISMM treatment.

Pre-infection results showed a significant increase in IFN-γ production by sheep PBMC 14 to 21 days after ISMM administration when cultured with live tyrpanosomes, while cultures from the control group were negative. No significant amounts of IFN-γ were detected in all groups during the infection period. ISMM prophylaxis suppressed polyclonal lymphocyte proliferation *in vivo*. Increases in B-cells in the control and treated groups 14 to 21 days after infection were significantly higher than in the prophylactic group. A significant decrease in $CD4^+$ T-cells was recorded in the control and treated groups 14 to 21 days post infection, while in the prophylactic group no changes were observed. $CD8^-$ T-cells increased only after treatment. The ratio of $CD4^+$:$CD8^+$ T-cells significantly dropped 21 days after infection in the control and treated groups, while it increased in the prophylactic group. There were no significant differences in $CD5^+$ and $\gamma\delta^+$ T-cell responses. Trypanosome specific IgG antibodies in serum of the prophylactic group were significantly higher than those in the control, while they were absent in the treated group. Following BCG vaccination, lymphocyte proliferation *in vivo* was suppressed in the ISMM treated group. The ratio of $CD4^+$:$CD8^+$ T-cells was higher in the ISMM group than in the control. Also ISMM, prevented a decrease in the percentage of $CD4^+$ T cells and suppressed polyclonal $CD5^-$ T cells and B cell expansion. Little or no differences were observed on $\gamma\delta^-$, $CD8^+$ T cells, and PPD skin test. ISMM was trypanostatic *in vitro* and T cell suppression decreased the prepatent period in prophylactically treated mice.

In conclusion, ISMM prophylaxis modified cellular and antibody responses to *T. congolense* infection probably via the IFN-γ and IL-12 feedback mechanisms. This immunomodulation enhanced prophylaxis and is not specific for trypanosome antigens since similar changes were observed following BCG vaccination, although the end result of an infection may depend on the type of host animal and nature of antigen.

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CHAPTER 1

CHAPTER 1
GENERAL INTRODUCTION

Animal trypanosomiasis is one of the most important diseases affecting livestock productivity in Africa. Approximately 30% of Africa's estimated cattle population of 160 million and a comparable percentage of small ruminants are at risk of trypanosomiasis (ILRAD 1993/4). In Zambia, more than 60% of an estimated 2 million cattle population are at risk of the disease (Mumba and Chizyuka 1987). Morbidity and mortality caused by trypanosomiasis seriously affects or in some regions precludes, the rearing of livestock especially cattle. Infections also occurs in many wildlife species such as warthog, and bush pig but are usually subclinical thereby providing an extensive reservoir of infection from which domesticated livestock can become infected. Partly as a result of this disastrous situation Africa produces about 70 times less animal protein per unit area than Europe (Allsopp, Hall and Jone 1985). Losses in milk production, milk yield and tractive power and costs of running programmes to try to control the disease are estimated at US$500 million per year. Adding to this figure estimates of the lost potential in crop production which depends on draught power, increases the cost to US$5 billion each year (ILRAD 1993/4).

The aetiological agents of the disease are protozoan parasites belonging to the genus *Trypanosoma*. There are many different species of pathogenic trypanosomes most of which are transmitted to animals by arthropod vectors through either cyclical or noncyclical (mechanical) means in different ways. *T. equiperdum* which causes a venereal disease affecting mainly horses and related animals is the exception, where the parasite is transmitted *in coiton*.

Non cyclical transmission is effected by a simple mechanical transfer of the trypanosomes from one mammalian host to another by the interrupted feeding of biting insects like the *Stomoxys* and tabanids. In domestic animals *T. brucei evansi* is the most important and is widely distributed in Africa, Asia, Central and South America. In cyclical transmission the arthropod is a necessary intermediate host in which the trypanosomes undergo a period of development involving a series of morphological transformations before forms infective for the mammalian host, called

metacyclic trypanosomes, are produced. Cyclical transmission can further be divided into *Salivarian* and *Stercorarian* groups on the basis of their life cycle. The *Salivarian* group is restricted to Africa south of the Sahara, and includes all trypanosomes transmitted by tsetse flies (*Glossina*) such as *T. congolense*, *T. vivax*, *T. brucei* and *T. simiae*. Multiplication of the tsetse-transmitted trypanosomes takes place in the intestinal tract and the proboscis of the fly so that the infection of the mammalian host takes place while the fly is feeding. In the *Stercorarian* group, multiplication and transmission occurs in the gut of the vector, and the infective forms migrate to the rectum so that the metacyclic trypanosomes are passed with the faeces on to abrasions in the skin of a mammalian host. In domestic animals this group is relatively non-pathogenic and is transmitted by tabanid flies and sheep keds. The *Stercorarian* group contains *T. cruzi*, which is the cause in human of the serious Chagas Disease in South America, transmitted in faeces of triatomine bugs. In domestic animals the *Stercorarian* group is important only because of interferences with serological tests.

In Africa, the tsetse-transmitted trypanosomes (*T. congolense*, *T. vivax*, *T. b. brucei* and *T. simiae*) are the most important span of pathogenic trypanosomiasis. An area of approximately 10 million square kilometres of Africa between latitudes 14°N and 29°S (Urquhart and Holmes 1987) is infested by tsetse flies carrying these parasites (Figure 2.2). Although the infection rate in *Glossina* is low, ranging from 1 to 20% of the flies, each fly is infected for life. Two groups of tsetse-transmitted trypanosomes can be distinguished: the haematic and humoral group. The haematic group, which includes *T. congolense* and *T. vivax*, comprises parasites which are confined to blood and the lymphatic system. This group is characterised by the major pathology being anaemia. The humoral group, is characterised by tissue damage and includes *T. b. brucei*, *T. b. evansi* and the human pathogens *T. brucei rhodesiense* and *T. brucei gambiense*) are found in body cavities and intercellular tissue, and parasitaemia is observed mainly in terminal stages (Losos and Ikede 1972). The severity of the disease depends on a number of factors including the trypanosome species, strain variant, and species of host. The infection can vary from acute, as in *T. simiae* in pigs and *T. b. evansi* in camels to mild or almost inapparent, as in *T. b. brucei* in cattle. In

West Africa *T. vivax* produces the most important form of trypanosomiasis. *T. congolense* causes serious disease and death in cattle, horses, sheep and dogs, and is most prevalent in East, Central, and Southern Africa. At the site of tsetse fly bite metacyclic trypanosomes multiply in the skin producing within a few days a raised cutaneous oedematous swelling called a chancre (Mwangi 1991). Thereafter, trypanosomes enter the blood stream, multiply and a detectable parasitaemia becomes apparent in the peripheral blood within 7 to 14 days post infection. The parasitaemia may persist for many months, though its level may fluctuate due to the immune response of the host. The major clinical signs in ruminants are anaemia, generalised enlargement of the superficial lymph nodes, fever, lethargy and progressive loss of bodly condition. *T. congolense* infection is typically chronic, lasting for several months, and usually leads to death in susceptible untreated animals (Boyt 1985).

There is considerable evidence indicating that antibodies are important in the development of immunity against trypanosomes. Antibodies capable of *in vitro* neutralisation, agglutination, lysis and *in vivo* protection against variant specific surface antigens (VSSA) have been detected in the IgG and IgM fractions of cattle serum (Luckins 1976; Musoke *et al* 1981). IgG antibodies against common antigens have also been demonstrated (De Raadt 1974). However, there is no evidence that antibodies against common antigens play an important role in protection but may be involved in pathogenesis through the formation of immune complexes. The crucial role of antibodies in providing protection has been further supported by passive immunisation studies in mice. There are many reports that mice can be protected against *T. b. brucei* by immune serum or by transfer of B cell enriched fractions of spleen cells, but not with T cell enriched fraction (Seed 1977; Takayanagi and Enriques 1973, Takayanagi and Nakatake 1975; Campbell and Phillips 1976). No passive immunisation experiments in ruminants have been reported.

T cell deficient congenitally athymic nude (nu/nu) mice infected with *T. b. brucei* had lower parasitaemia and survived longer than intact control mice (Campbell, Esser and Phillips 1978). In contrast, congenitally nude (nu/nu) mice infected with *T. congolense* had higher parasitaemia and their survival time was shorter than intact

control mice (Morrison *et al* 1978). Results obtained from *T. brucei* infections imply that T cell mediated responses against trypanosomes contribute to pathology, while those from *T. congolense* infection imply that they are important for an effective control of trypanosomiasis. In addition, specific delayed type hypersensitivity reactions against trypanosome antigens have been detected in rabbits infected with *T. b. brucei* (Tizard and Soltys 1971), mice injected with formalin fixed *T. b. rhodesiense* (Finerty, Krehl and McKelvin 1978), and cattle infected with *T. congolense* (Emery and Moloo 1980).

Although the immune system can be effective at clearing parasites, the effect may be short lived because trypanosomes have a very sophisticated way of evading the host's immune system through the process of antigenic variation. Extensive studies and reviews have been written on the subject of antigenic variation (Cross 1990; Gray and Luckins 1976; Doyle 1977). Briefly, antigenic variants that arise during infection are unique biochemically and serologically. The immune response to each specific variant may clear parasites expressing that particular variable antigen epitope but provides no protection against trypanosomes bearing different surface coats, thereby allowing new variants to appear. This results in a fluctuating parasitaemia. Antigenic variation is one of the main reasons why artificial immunisation has not been successful (Duxbury *et al* 1972; Wellde *et al* 1973). The other reason is that the nature and pattern of the actual protective immune mechanisms is poorly understood despite the fact that much of work on antibody responses and antigenic variations has been done. This may partly be due to the fact that until recently, cellular responses have not been investigated in great detail.

Because immunity to trypanosome infection has a very strong antibody dependence, until recently very little had been done to investigate cell-mediated immune responses to trypanosomiases. Studies in cattle and sheep infected with *T. congolense* have shown significant changes in $CD4^+$, $CD8^+$, $CD5^+$ (Williams *et al* 1991; Mwangi 1991) and $\gamma\delta^+$ (Flynn and Sileghem 1991) T cell responses. Effector T cells may have no direct role in destroying trypanosomes, but significant changes in regulatory cytokine

and T cell function might have a profound effect on the function of the immune system during infection, hence the need to investigate them in more detail.

Control of trypanosomiasis is achieved by control of or eradication of the vector, treatment of infected animals and breeding of trypanotolerant animals, vector, and parasite control. Usually integrated control programmes are employed.

Vector control methods that are currently in use include: aerial and/or ground spray of residual insecticides such as deltamethrin, application of insecticides on the body of animals (pour-ons) and the use of traps or targets which involves the spraying of an insecticide onto a piece of cloth (target) coupled with a tsetse attractant, acetone and 1-octen-3-ol (Allsopp, Hall and Jone 1985). Due to environmental concerns, methods aimed at tsetse eradication have been abandoned in many areas, and since the existing vector control methods are expensive and protection is not full proof the risk of infection always exists.

Trypanotolerance means reduced susceptibility to trypanosomiasis and denotes an inherited biological characteristic enabling animals to live, breed, grow and survive in a naturally infected environment without showing signs of disease after harbouring the trypanosomes (Murray, Morrison and Whitelaw 1982; Camus 1981). Trypanotolerance is found in cattle such as the N'dama (*Bos taurus*), sheep, goats and some pony types (Chabeuf 1983). Field studies by Njogu *et al* 1985 have demonstrated that even among Zebu (*Bos indicus*) cattle which are generally considered to be susceptible, some types of Borans can be more tolerant than others. However, breeding for trypanotolerance alone is not a sufficient control programme and in general, most trypanotolerant *Bos taurus* cattle have a low productivity compared to susceptible *Bos indicus*.

Parasite control measures involve diagnosis and treatment as well as chemoprophylaxis. Treatment of trypanosomiasis in cattle depends on mainly three drugs; diminazine, homidium and isometamidium. Diminazine and homidium are used

as therapeutic agents and only isometamidium is used for both therapeutic and prophylactic purposes.

ISMM has a high activity against *T. congolense*, and *T. vivax*, but low against *T. brucei* and *T. evansi* probably because their presence in tissues makes them less accessible. The suggested mode of action is through interference with parasite DNA synthesis by intercalating between base pairs: ISMM has been shown to inhibit both DNA and RNA polymerases. However, since it affects both trypanosome and mammalian DNA in the same manner, the selective toxicity must be elsewhere. ISMM is usually administered by intramuscular injection at 0.5 mg/kg and 1 mg/kg for therapy and prophylaxis respectively. It binds to tissue macromolecules and also precipitates proteins causing inflammation and necrosis with leukocyte and later macrophage infiltration at the injection site. Accumulated concentrations are highest at the injection site followed by the liver, kidney and spleen (Kinabo and Began 1988a). These sites provide depots for long term release. After intramuscular administration of 0.5 mg/kg Kinabo and Bogan (1988a) measured an average maximum serum concentration of 20 ng/ml in cattle 30 to 45 minutes after administration. Braide and Eghianruwa (1980) reported higher mean plasma levels of 2.17 μg/ml after 24 hours in goats. In camel 24 hours following intravenous injection of 0.5 mg/kg ISMM the mean maximum plasma level was 0.7 μg/ml (Ali, Hassan and Malik 1984). However, protection is longer with intramuscular than with intravenous injection. ISMM is widely used for prophylaxis and has been shown to protect animals against trypanosome infection for periods up to 6 months (Fairclough 1963; Pinder 1984; Eisler *et al* 1994).

Prophylactic failure is the major problem associated with the use of ISMM. The manufacturers recommends a 3 month dosing interval. At this frequency of use the drug is generally considered to be cost effective. However, it is very common for a considerable number of animals to become infected by the end of one month even after careful administration (Fairclough 1963; Pinder 1984; Eisler *et al* 1994). A dosing interval of less than 3 months generally ceases to be cost effective and increases treatment costs or animal losses. In order to correct the problem of

prophylactic drug failure, a substantial understanding of protective mechanisms involved is vital. The mechanism of protection when ISMM plasma levels are low or undetectable is still not well known. Whitelaw *et al* (1986) reported that only drug residues are important and not the immune system because they were unable to detect trypanolytic antibodies in treated animals. However, the fact that they were unable to detect trypanolytic antibodies in serum does not rule out the involvement of other aspects of the immune system.

A number of observations have been made which suggest that the drug can be effective even when plasma levels are very low or undetectable (Kinabo 1993). For instance following intramuscular or intravenous administration to cattle, plasma levels can fall below 6.1 ng/ml by the end of one month (Kinabo and Bogan 1988a; Eisler *et al* 1994) but still offer protection against challenge by sensitive trypanosome strains (Eisler *et al* 1994). However, *in vitro* sensitivity tests have shown that exposing sensitive trypanosomes to 5 ng/ml of ISMM for less than 10 minutes (Sutherland, Mounsey and Holmes 1991), 10 ng/ml for less than 24 hours (Kaminsky, Chuma and Wasike 1994) and 10 ng/ml for less than 48 hours (Gray and Peregrine 1993) could not completely eliminate the parasites or prevent infection. This implies that the direct minimum effective concentration (MEC) for ISMM should be in the range of 5-10 ng/ml. What accounts for the observed protection when plasma levels are below that which is considered to be the MEC (5 ng/ml) is still a mystery. Nevertheless, only free (unbound) drug in serum can be absorbed by the parasite and therefore, affect its growth. There are a number of views that try to explain the effectiveness of ISMM at undetectable levels. They include: special trypanosomal uptake mechanisms which lead to lethal drug levels inside the parasite; the level of tsetse challenge; the possible production of active metabolites (Kinabo 1993) and boosting of the immune responses. Out of all these, special uptake mechanisms, and level of tsetse challenge have been studied and found to have no significant effect. Information on putative active metabolites is not yet available. No trypanolytic antibodies have been detected in prophylactically treated cattle infected with *T. congolense* six months later (Whitelaw *et al* 1986; Peregrine *et al* 1988). However, there is no existing

information on the possible enhancement of T cell immune responses in prophylactically treated animals.

ISMM is trypanostatic and not trypanocidal which makes it more likely to be dependent on the immune system for its effectiveness. Culturing of trypanosomes, *in vitro* in the presence of 5 ng/ml ISMM for less than 10 minutes (Sutherland, Mounsey and Holmes 1991), 10 ng/ml for less than 24 hours (Kaminsky, Chuma and Wasike 1994) and 10 ng/ml for less than 48 hours (Gray and Peregrine 1993) could not completely eliminate the parasites or prevent infection. Yet at concentrations of ISMM less than 6 ng/ml, the establishment of a *T. congolense* infection was prevented (Eisler *et al* 1994). This makes potentiation of some aspects of the immune system a possible mechanism of action. However, interactions of the drug with the immune system of both normal and trypanosome infected animals is not well known. Knowledge of this interaction could be very useful in understanding the mechanism of prophylaxis as well as the development of drug resistance. This would in turn lead to a better utilisation of the drug to obtain the maximum benefit and could also probably pave the way for the development of other much needed prophylactic drugs.

In a trypanosmiasis endemic area, animals are usually found in different disease states when a chemoprophylactic agent like isometamidium is administered. Some will be completely free of the infection while others might be already infected. The drug will therefore act as a prophylactic agent for those animals that would not be infected and act as a therapeutic agent for those already infected. As a result any possible drug modulation of immune responses in an endemic population is expected to be different depending on the state of the disease the animal might be in at the time of administration It is therefore, necessary to study immune responses in the prophylactic and treated groups and make comparisons with the control group in order to draw a meaningful relationship with the situation in the field.

The objective of this project was to investigate effects of isometamidium ISMM, on immune responses to *T. congolense* infection in order to understand mechanisms of prophylaxis so that the monitoring of prophylaxis can be improved in the field. (1) the

efficacy of ISMM on *T. congolense in vitro* and *in vivo* was examined. (2) effects on sheep peripheral blood mononuclear cell (PBMC) phenotypes, proliferation, and IFN-γ production were investigated. (3) effects on IL-12 and IFN-γ production by mice splenic cells were studied. (4) effects on sheep PBMC phenotypes following BCG vaccination in sheep were also investigated in order to establish whether effects of ISMM could be specific to trypanosome antigens.

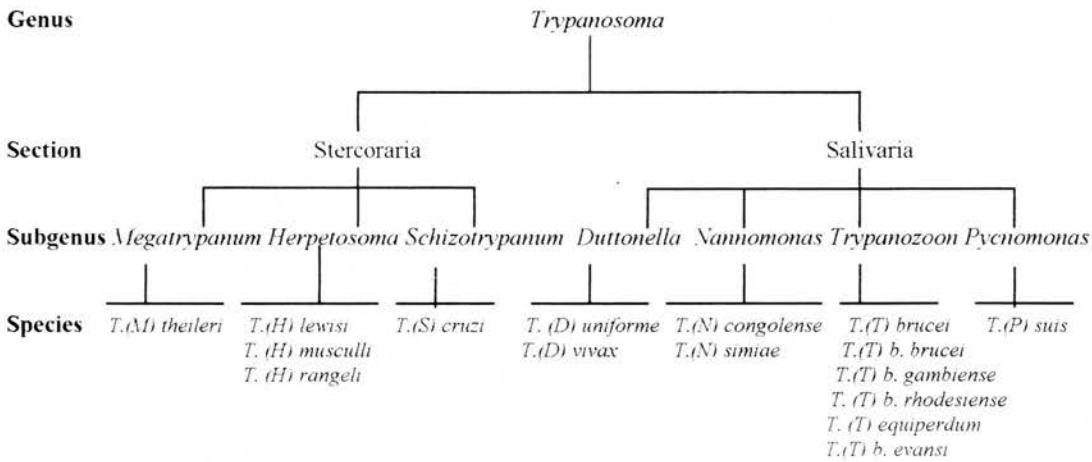
CHAPTER 2

CHAPTER 2
LITERATURE REVIEW

2.1 NOMENCLATURE AND CLASSIFICATION OF TRYPANOSOMES

Trypanosomes are eukarotic organisms found in the blood or tissues of vertebrate animals and the gut of arthropods and other organisms (Hoare 1972). Members of the genus *Trypanosoma* (Figure 2.1) are elongated protozoa which are 8 - 39 μm long. They have a flagellum which runs to the anterior end of the body and is attached to the body by an undulating membrane. The flagellum functions as a locomotion organelle and may be free in some species. Staining with Giemsa or most other conventional stains shows a kinetoplast at the base of the flagellum and a centrally located dark staining nucleus (Vickerman *et al* 1980).

Figure 2.1 Classification of the genus *Trypanosoma*



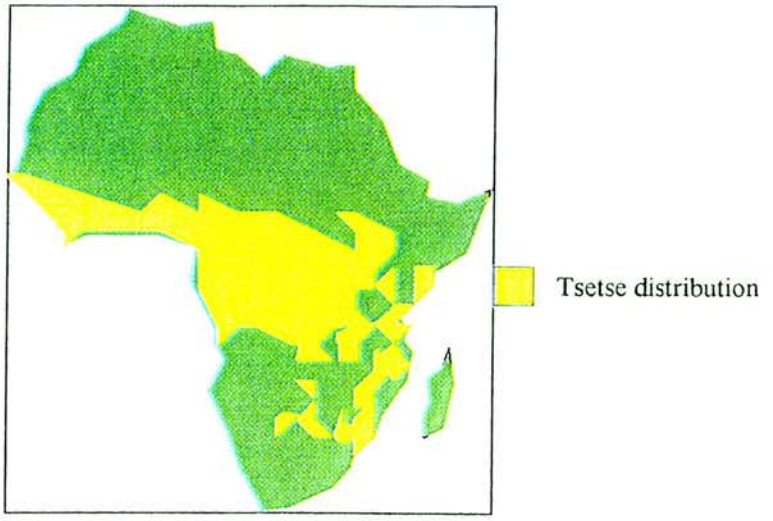
The *Salivarian* group includes all trypanosomes transmitted by tsetse flies (*Glossina*) in addition to *T.(T) equiperdum*, which is transmitted during coitus in horses and related species, and *T.(T) b. evansi* which is transmitted mechanically by tabanid flies. Multiplication of *Salivarian* trypanosomes takes place in the intestinal tract and the proboscis of the fly so that the infection of the mammalian host takes place while the

fly is feeding. Mechanical transmission occurs by a simple transfer of the trypanosomes from one mammalian host to another by the interrupted feeding of biting insects like the *Stomoxys* and tabanids. In this group *T. brucei evansi* is the most important and is widely distributed in Africa, Asia, Central and South America. Trypanosomes of the *Stercorarian* group multiply in the gut of the tsetse fly, and the infective forms migrate to the rectum so that the metacyclic trypanosomes are passed with the faeces on to abrasions in the skin of a mammalian host. *Stercorarian* trypanosomes are relatively non-pathogenic in domestic animals and are important only because of interference with serological tests especially *T. (M) theileri* which is transmitted by tabanid flies and sheep keds. The group is important in human because it contains *T. cruzi*, which is the cause Chagas Disease in South America, transmitted in faeces of triatomine bugs. However, in addition to the typical *Salivarian* or *Stercorarian* pathways of infection any trypanosome can also be transmitted mechanically.

T. congolense is the major cause of animal trypanosomiasis in Central, East and Southern Africa (Fiennes 1950; Mulligan 1970), while by *T. vivax* is the major cause in West African trypanosomiasis (Losos and Ikede 1972). The disease affects domestic ruminants, horses and pigs, while wildlife such as warthogs are reservoir hosts. Since *T. congolense* is tsetse transmitted, the distribution of the disease is limited to tsetse infested areas of Africa (Figure 2.2).

Three major groups of tsetse flies (*Glossina* species) are recognised: *morsitans*, *palpalis* and *fusca*. *G. morsitans* is the major vector of animal trypanosomes because their habitat (Savannah woodland) is shared by livestock. *G. palpalis* is the second most important because it inhabits the riverline but is not as widely distributed as *G. morsitans*. *G. fusca* inhabits the forest, and although they are very efficient at transmitting trypanosomes they are the least important because livestock rarely graze in the forest (Jordan 1986).

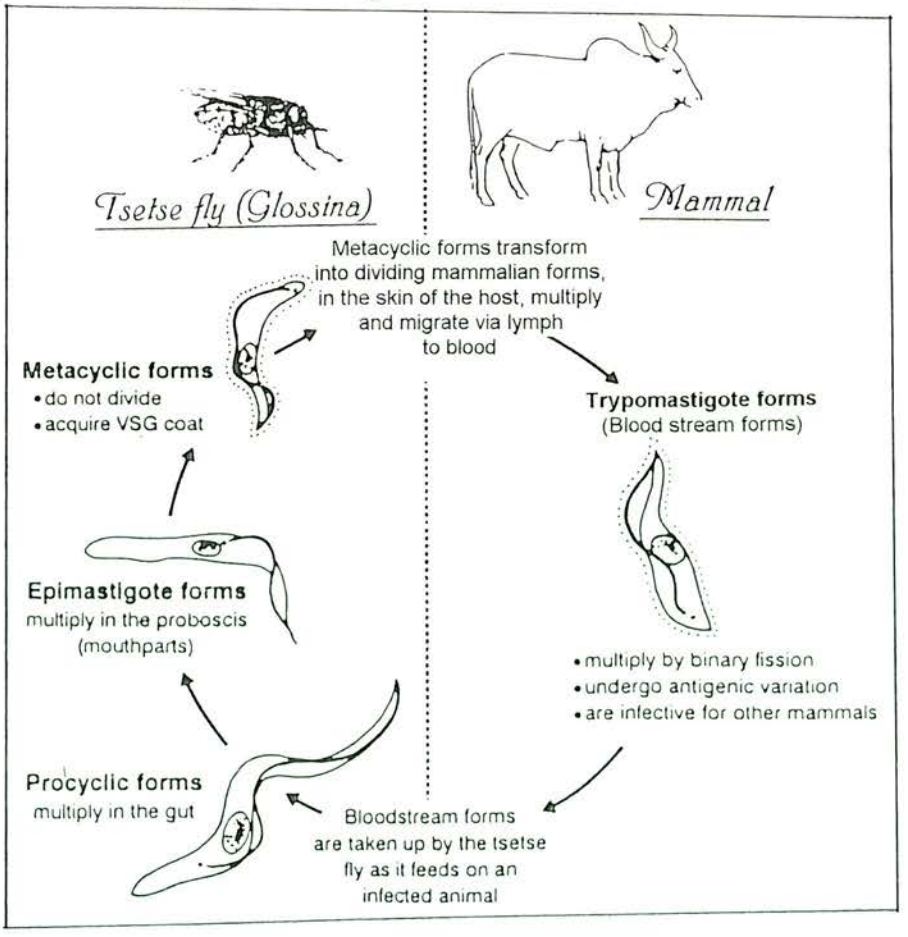
Figure 2.2 Map of Africa showing the distribution of tsetse flies, the vector for trypanosomiasis



2.2 LIFE CYCLE OF *T. CONGOLENSE*

The life cycle and survival of *T. congolense* requires the tsetse fly (*Glossina*) and a mammalian host and has been studied extensively by many researchers (Hoare 1972; Evans, Ellis and Stomford 1979) (Figure 2.3).

Figure 2.3 Life cycle of *T. congolense*



Procyclics, epimastigotes, and metacylics (infective forms) are found in the tsetse fly. Trypanosomes ingested by the fly begin their development in the endopeirtrophic space of the midgut. Blood stream forms lose their surface coat and morphologically become elongated procyclics which have the kinetoplast positioned posterior to the nucleus. Procyclics escape through the posterior end of the peritrophic membrane and migrate to the proventriculus where they enter the endoperitrophic space. They migrate through the oesophagus to the proboscis where they attach to the walls of the labrum and transform into epimastigotes. Epimastigotes have no surface coat and their kinetoplast is positioned anterior to the nucleus. Epimastigotes then transform into infective metacyclics which migrate to the hypopharynx. Metacyclics are morphologically similar to blood stream forms and have a surface coat but are non-dividing. Completion of all stages in the tsetse fly can take 19 to 53 days (Hoare 1972) or 7 to 40 days (Nantulya, Doyle and Jenni 1978) depending on the type of isolate and host used in the study. When tsetse flies feed on mammalian hosts they inoculate a mixture of procyclics, epimastigotes and infective metacyclics. Metacyclics transform into dividing mammalian forms (Dwinger *et al* 1987) and migrate to the lymphatics and blood stream where they multiply continuously as trypomastigotes by binary fission (Dwinger *et al* 1987; Akol and Murray 1982). The cycle is completed when tsetse flies ingest trypomastigotes when they feed on the host, and trypomastigotes develop into procyclics in the tsetse fly.

2.3 *IN VITRO* PROPAGATION OF *T. CONGOLENSE*

Almost all life cycle stages of *T. congolense* and other trypanosome species can be propagated *in vitro*. Gray *et al* (1981) reported the initial success in the production of culture-derived metacyclic forms of *T. congolense*. Continuous cultures of procyclic, epimastigote and metacyclic forms have been maintained in Eagles minimum essential medium supplemented with 20% foetal calf serum and 2% of 200mM L-glutamate in plastic culture flasks at 28°C (Gray and Luckins 1979; Ross *et al* 1985) without a feeder layer such as dermal collagen (Hirumi and Moloo 1982). Culture-derived metacyclics retain the antigenic characteristics of their parent isolate and can be infective to rabbits (Luckins, Rae and Gray 1981) and cattle (Akol and Murray 1985).

Continuous cultures of blood stream (trypomastigotes) forms of *T. congolense* is also possible in a 1:1 supplemented mixture of RPMI 1640 and IMDM (Ross and Taylor 1990).

2.4 PATHOGENESIS OF TRYPANOSOMIASIS IN DOMESTIC ANIMALS

Acute, subacute and chronic forms of the disease have been reported, with severity varying according to the susceptibility of the host (Losos *et al* 1973; Griffin and Allonby 1979). However, the chronic form, which persists for longer than three months, is the most common (Morrison, Murray and McIntyre 1981). The pre-patent period varies from 7 to 25 days in sheep and cattle (Griffin and Allonby 1979). Clinical signs include; enlarged superficial lymph nodes, fluctuating fever, pale mucous membranes, loss of body condition, infertility and abortions (Wellde *et al* 1974; Griffin and Allonby 1979, Mutayoba, EcKersall and Cestnik 1995). The major pathological signs include; anaemia, enlarged lymph nodes, spleen and liver as well as inflammatory changes in the heart, kidneys, lungs and testicles (Valli and Forsberg 1979; Kaaya and Oduor-Okelo 1980). However, anaemia is the most significant and probably the primary cause of death in most domestic ruminants (Mamo and Holmes 1975; Morrison, Murray and Akol 1985).

2.5 IMMUNE RESPONSES TO TRYPANOSOMES

Infected animals show a great variation in the immune responses against trypanosomes. When cattle are infected with a single serodeme they may sometimes be able to eliminate the parasites and recover from the infection (Morrison, Murray and Akol 1985). Certain breeds of cattle e.g the N'dama of West and Central Africa are resistant to trypanosomiasis because they have a greater capacity to control the infection (Murray, Morrison and Whitelaw 1982). A major feature of immune responses in both laboratory and domestic animals is the change in cellularity and architecture of various lymphoid organs (Mansfield 1982). These changes are expected to interfere with the normal immune functions because of the important role played by lymphocytes and their interaction with other cells of the immune system.

2.5.1. HUMORAL IMMUNE RESPONSES TO TRYPANOSOMES

After infection with trypanosomes, there is an elevated level of IgM antibodies in most mammals such as cattle, sheep, rodents, humans, monkeys and bush buck (Houba, Brown and Allison 1969; Luckins 1972; Mackenzie, Boyt and Nesham 1979; Hudson *et al* 1976). Most of these IgM antibodies produced are not specific for the particular serodeme (Hudson *et al* 1976; Terry, Hudson and Faghihi-Shirazi 1980; Corsini *et al* 1977; Musoke *et al* 1981). The ability of the host to control the infection is however, dependent on the ability to produce IgM antibodies (MacAskill *et al* 1983), although IgG antibodies are now considered to play a significant role in parasite clearance. Antibody mediated lysis (trypanolysis) occurs rapidly *in vitro* in the presence of complement (Barry and Vickerman 1977). Most studies have demonstrated that complement is required for immune lysis of trypanosomes to take place *in vitro*, however, the role of complement in immunolysis *in vivo* is controversial since trypanosome infected animals are hypocomplementaemic probably as a result of complex formation and direct activation by trypanosomes (Mansfield 1981). It is more likely that clearance of antibody coated trypanosomes occurs primarily by phagocytosis and there is a considerable evidence in support of this view. *In vitro* studies have shown that *T. brucei* and *T. gambiense* are ingested by macrophages in the presence of immune serum (Lumsden and Herbert 1967). *In vivo* evaluation of the role of opsonisation and macrophage uptake of radiolabeled trypanosomes showed that whereas labelled parasites remained in circulation of normal mice, they rapidly disappeared from blood of immune animals. In the immunised mice the liver was found to be the principal site of uptake, removing over 50% of trypanosomes within 15 minutes of an intravenous injection of labelled parasites (Holmes *et al* 1979; MacAskill *et al* 1980). The possibility that immune lysis was a pre-requisite to phagocytosis of trypanosomes was investigated *in vivo* by measuring trypanosome clearance ability of passively immunised C5-deficient and C3-depleted mice. Results of this experiment showed that C5-deficiency which is necessary in complement mediated lysis did not affect immune clearance, while reduction in C3 which is essential for full opsonic activity prevented immune clearance in passively immunised mice (MacAskill *et al* 1980).

The specific antibody response to trypanosomes is highly related to the variable surface glycoprotein (VSG). Very few antibodies to non-variable antigens are detected. The primary antibody response to trypanosome VSGs in laboratory and domestic animals usually reaches a peak 7 - 14 days after infection, then decreases slowly for more than 100 days (Herbert *et al* 1980; Roelents and Pinder 1984). The actual duration of protection for the particular serodeme in cattle varies from 35 days (Wiesenhutter 1970) to 14 months (Wellde *et al* 1973). There are conflicting reports about the dominant secondary antibody isotype involved. Some reports show that IgM still dominates (Oka *et al* 1981) while others show an increase in IgG isotype (Roelants and Pinder 1984; Musoke *et al* 1981). The antibody isotype present at any particular time is important since different isotypes have different binding affinities.

2.5.2: CELL-MEDIATED IMMUNE RESPONSES TO TRYPANOSOMIASIS

Very little work on cell-mediated immune responses to trypanosomes was performed before the 1980s because of strong evidence that immunity was antibody mediated. However, the little evidence that was available then suggested that cell-mediated immune responses were either absent or played no protective role (Campbell, Esser and Phillips 1978; Clayton, Ogilvie and Askonas 1979; Askonas *et al* 1979; Takanagi and Nakatake 1975). However, effector T cells may have no direct role in destroying trypanosomes, but significant changes in regulatory T cell function might have a profound effect on the function of the immune system during infection. Evidence in support of cellular immune responses include results from studies that have shown *in vitro* proliferation of mononuclear cells occurring 5 - 10 days after priming and decreasing after 15 days (Gasbarre, Hugh and Louis 1981; Flynn *et al* 1991; Lutje *et al* 1995), when antibody levels would be high enough to control the infection. The decrease in proliferative responses observed 15 days onwards may be a result of a negative feedback to reduce adverse effects that would result from a hyperstimulated immune system. Further evidence for cellular response playing a significant role in the control of trypanosomiasis came from studies in rodents infected with trypanosomiasis which showed a delayed type hypersensitivity reaction to trypanosome antigens

(Tizard and Soltys 1971). It is expected that if cellular responses were not involved in the control of the disease, no delayed type hypersensitivity reactions could be observed.

A skin reaction called chancre which develops at the site of inoculation of the metacyclic trypanosomes is also a cellular immunological reaction which usually starts within 3 - 5 days due to multiplying trypanosomes that produce an inflamed oedematous lesion several centimetres in diameter and persists for 10 - 15 days (Gray and Luckins 1980; Akol and Murray 1982; Mwangi, Hopkins and Luckins 1990). Cell types involved in the reaction are initially inflammatory then immunological, with a progressive increase of lymphoblasts and plasma cells (Mwangi 1991). A chancre is a feature of naïve animals and those treated with drugs before it develops and subsequently challenged. Chancre is not observed during secondary infections or in animals treated with anti-trypanosomal drugs after it had developed (Wilson *et al* 1971; Luckins 1986). Consequently, it is not common to come across animals with chancres in trypanosome infected areas unless they had just been introduced from a trypanosomiasis free area.

T cell phenotype responses have been demonstrated in sheep (Mwangi 1991) and cattle (Williams *et al* 1991; Lutje *et al* 1995). In these studies, animals resistant to trypanosomes showed significant increases in the ratio of $CD4^+:CD8^+$ T cells indicating an increase in helper T cell activity which up-regulates immune responses. On the other hand trypanosome infections can influence cytokine production such as; suppression of IL-2 production in mice infected with *T. congolense* (Mitchell, Pearson and Gauldie 1986), and *T. brucei* (Sileghem *et al* 1987) and suppression of IL-2 and IL-2R expression in cattle (Sileghem *et al* 1989b; Lutje *et al* 1995). Induction of IFN-γ production by *T. brucei* infection in mice which leads to suppression of mononuclear cell proliferation (Darji *et al* 1991; Sileghem, Darji and De Baetselier 1991; Olsson *et al* 1991; Bakhiet *et al* 1996) and *T. congolense* infection in cattle where highest levels were detected 11 days post infection onwards (Lutje *et al* 1995; Taylor, Lutje and Mertens 1996). Taylor *et al* (1996) also demonstrated an up-regulation of IL-10 in

cattle infected with *T. congolense*. Suppression of IL-2 production and IL-2R expression and an increase in IFNγ production in mice seem to contribute to the pathogenesis since IFNγ appears to promote parasite growth and suppress mononuclear cell proliferation (Olsson *et al* 1991; Bakhiet *et al* 1996). It is well documented that cytokines are very important in regulating immune responses (Trinchieri 1997) and detailed effects of these cytokine changes is discussed below in section 2.6.2 under the heading of 'suppression of specific cell mediated responses'.

2.5.3 Antigenic variation

Immunity to trypanosomes is rapid and strong and is mediated by antibody response against the variant surface glycoproteins (VSG) (Murray and Urquhart 1977), but because of antigenic variation protection does not last long (Vickerman and Barry 1982; Morrison, Murray and Akol 1985). Antigenic variation by trypanosomes is a way of evading the defence system of the host and is manifested by the sequential expression of VSGs which form the surface coat covering the trypanosome body and flagellum (Vickerman 1969; Cross 1990). The VSG is a unique feature of trypomastigote (blood stream) forms and metacyclic (infective) forms of trypanosomes which come in contact with host defences (Vickerman 1969). After an infection the animal elicits an immune response against the invading variant antigen types (VATs) but the trypanosome has the genetic ability to recognise danger and generate a different VSG that is not recognised by the mounted immune response (Cross 1978). The new VATs are seen by the animal as if they were a fresh infection and it takes time before an effective immune response against the new variant would develop and by then the parasites would be launching another distinct variant, resulting in a persistent fluctuating parasitaemia (Vickerman 1978). Fluctuating waves of parasitaemia results from immunological destruction by the host of trypanosomes expressing VATs against which an immune response has already been mounted (Esser, Schroenbechler and Gingrich 1982).

The switch from one VSG type to another is a consequence of sequential activation of as many as 1000 different VSG genes present in trypanosomes (Gibson, Marshall and

Godfrey 1980) and is independent of antibody pressure since it has been demonstrated that antigenic variation occurs *in vitro* in the absence of antibodies (Doyle *et al* 1980; Luckins *et al* 1990). At present the factor responsible for triggering antigenic variation is not known, but considering that all *in vitro* culture systems contain animal serum (Ross and Taylor 1990) it is very likely that the triggering factor should be a constituent of animal serum. This is an area that needs to be investigated further as well as the effect of ISMM and other anti-trypanosomal drugs on antigenic variation both *in vitro* and *in vivo*. It is possible that any agent that inhibits antigenic variation would be useful for prophylaxis even without having trypanocidal effects since immunity to any particular serodene is strong enough to eliminate non-variant trypanosomes.

During the life cycle of trypanosomes, antigenic variation stops after trypomastigotes have been ingested by the tsetse fly and *in vitro* when transformation to procyclics takes place (Ehlers, Czichos and Overath 1987). It starts again in the tsetse salivary glands when non-dividing metacyclics have been produced (Tetley *et al* 1987). However, the population of metacyclics is heterogeneous, with a small predictable number of VAT (Le Ray, Barry and Vickerman 1978). For instance, one strain of *T. congolense* contains only 12 metacyclic VATs and the number is also limited in *T. rhodesiense* (Turner *et al* 1988). Metacyclic VATs of *T. congolense* continue to be expressed in the mammalian host while new VATs are being produced in the chancre (Luckins *et al* 1990). In contrast those of *T. brucei* cease to be expressed after inoculation into the animal leaving only trypomastigote VATs (Barry, Crowe and Vickerman 1983). This means metacyclics of *T. congolense* are more susceptible to early immunological responses than those of *T. brucei*. In addition re-expression of metacyclic VATs by trypomatigote forms of trypanosomes is possible (Nantulya *et al* 1984) making immunisation with metacylic forms of trypanosomes an attractive area of research.

2.6 IMMUNOPATHOLOGY

2.6.1 Anaemia and tissue damage

A significant loss of red cells and a decrease in the packed cell volume usually corresponds to the duration of parasitaemia. Anaemia is less severe in animals that are resistant to trypanosomiasis such as the N'dama cattle (Dargie *et al* 1979). The aetiology of anaemia is multifactorial with the formation of trypanosome-antibody-red cell immune complexes playing a major role. Initially haemolysis occurs probably mediated by bioactive substances released by dying parasites (Haun 1975; Tizard *et al* 1978). These bioactive substances include enzymes, lipids, haemolysins, inflammatory substances and mitogenic factors (Tizard *et al* 1978; Olsson *et al* 1991; Bakhiet *et al* 1996) which have the potential to cause red cell damage and that of other tissues. This mechanism may be effective in rodents which develop acute infections with high parasitaemias but may not be that effective in chronic infections in ruminants which are characterised by low parasitaemia. Destruction of trypanosomes produces both soluble, and fragments of antigen which complex with several cells and tissues in the host. Later, anti-trypanosomal antibodies attach to produce cell-antigen-antibody complexes which are susceptible to phagocytosis. In the case of erythrocytes, phagocytosis of complexes with trypanosome antigens takes place in the spleen, liver, and lymph nodes and is probably the major cause of anaemia in ruminant infections (Mackenzie and Cruickshank 1973). However, during the chronic stages of infection anaemia is due to failure of haemopoiesis in the degenerated bone marrow (Dargie *et al* 1979).

Immune complexes in tissues may initiate acute and later chronic inflammatory responses which may be responsible for tissue damage and muscle wasting observed in chronic infections. The other possible cause of tissue damage is the activation by immune complexes for the release of pharmacologically active substances such as histamine and 5-hydroxyltryptamine (5-HT) which increase the vascular permeability causing tissue oedema (Richards 1965). However, tissue damage is a feature common in *T. brucei*, while anaemia is common in *T. congolense* infections.

2.6.2 Immunosuppression in trypanosomiasis

Trypanosomes suppress both the specific humoral antibody and cell mediated immune responses resulting in the establishment of the infection and predisposing the host to secondary infections (Parkin and Hornby 1930; Fiennes 1954). However, immunosuppression in cattle may not be as severe as in rodents (Morrison and Murray 1979; Masake and Morrison 1981).

Suppression of specific antibody responses

Antibodies specific for an existing trypanosome VAT continue to be produced throughout the infection although the degree of response becomes progressively depressed (Hudson and Terry 1979). In chronically infected mice there is progressive suppression of trypanosome specific IgM and IgG antibodies such that by the third wave of parasitaemia there are only very low levels of IgM and no detectable IgG (Sacks and Askonas 1980). In cattle antigen specific antibody responses were depressed in *T. brucei* infected (Nantulya *et al* 1982) and in *T. congolense* infected cattle (Morrison, Murray and Akol 1985). However, the decrease in variant specific antibodies may be due to the gradual elimination of the particular variant by the immune system leaving new variants which may not react with the antigen used in the assay which is based on the variant that was inoculated but would have changed. Hence, although a decrease in specific trypanosome antibodies is generally considered to be suppression, the consequences of antigenic variation indicates that it is probably due to the removal by the immune system of the invading VAT which is associated with the antibody assay.

Clonal B cell exhaustion as a result of polyclonal B lymphocyte activation by a non-specific trypanosome derived mitogen (Askonas *et al* 1979; Clayton, Ogilvie and Askonas 1979; Oka *et al* 1984) has been proposed as a mechanism leading to specific suppression of antibody responses. Evidence for this came from *in vitro* studies of antibody producing B cells. Normally high levels of IgM and IgG are observed when cells proliferate *in vitro*. They are seen to rise when they are first put in culture and decrease as the infection progresses, suggesting exhaustion of B lymphocytes (Corsini

et al 1977). Nevertheless, due to antigenic variation occurring *in vitro*, these findings may again not be relevant if the antibody assay relies on the invading VAT.

Other possible mechanisms leading to suppression of antibody activity that have been observed in cattle infected with *T. congolense* include; increased immunoglobulin catabolism and hypocomplementaemia (Nielsen *et al* 1978). Progressive hypocomplementaemia is the reason trypanolysis *in vivo* may not be an important effector mechanism since it requires the presence of complement.

Suppression of non-specific antibody responses

Depression of antibody responses to non-trypanosome antigens during the period of trypanosome infection is very important because it renders animals susceptible secondary infection. There is evidence that mice infected with *T. brucei* had reduced antibody responses specific to sheep red blood cells (Goodwin *et al* 1972; Wellhausen and Mansfield 1979; Jayawardena, Waksman and Eardley 1978). Also *T. congolense* infection lead to depressed secondary antibody response to polyvalent clostridial vaccine in cattle. This may lead to reduced success of vaccination programmes in trypanosomiasis endemic areas (Holmes *et al* 1974; Scott 1977). Rurangirwa *et al* (1983) also found a profound suppression of IgG_1, IgG_2 and IgM responses to *Brucella abortus* (S-19) vaccine in cattle chronically infected with *T. congolense*. However, studies that measured antibody responses in the first few weeks of infection with *T. congolense* or *T. vivax*, showed little or no suppression of non-specific antibody responses (Ilemobade *et al* 1982; Whitelaw *et al* 1979; Rurangirwa *et al* 1980). These results demonstrate that non-specific suppression of antibody responses occurs during the chronic stage of infection probably as a result of polyclonal B cell activation and exhaustion of cells of the immune system.

Suppression of specific cell-mediated responses

At present investigations on proliferative responses of peripheral blood mononuclear cells (PBMCs) is commonly performed *in vitro*. PBMCs from infected and treated cattle proliferate *in vitro* in response to stimulation with homologous ultrasonicated

trypanosomal antigens. This response was suppressed in animals with active infection (Emery *et al* 1980). In addition peripheral blood lymphocytes (PBLs) from *T. congolense* infected cattle proliferated *in vitro* in response to ultrasonicated trypanosomal antigens when PBLs were collected 3-7 days post infection but thereafter, the proliferation was suppressed but appeared after treatment (Morrison, Murray and Akol 1985). These results demonstrate that mononuclear cells from animals in a stage of active trypanosome infection are refractive to any further antigen specific proliferative stimulus. However, during the period of active infection, there is usually increased lymphocytosis *in vivo* (Mwangi 1991; Williams *et al* 1991) which implies that results from *in vitro* proliferative responses are inversely related to what goes on *in vivo*. Therefore, although a decrease in specific *in vitro* proliferation during the period of active trypanosome infection is generally considered to be suppression, it is not truly immunosuppression *in vivo*.

In cattle and sheep infected with *T. congolense* there are changes in lymphocyte subsets such as depletion of $CD4^-$,$CD8^-$ and $\gamma\delta^-$ T cells in susceptible Boran cattle which required treatment while only $CD8^-$ T cells were depleted in trypanotolerant N'dama cattle which self cured (Williams *et al* 1991). In trypanotolerant N'dama cattle a decrease in the proportion of $CD8^-$ T cells with a constant proportion of $CD4^-$ T cells implies an increase in helper T cell activity resulting in an up-regulation of immune responses. On the other hand, a decrease in both $CD4^-$ and $CD8^-$ T cells indicates that there was suppression of helper T cell activity in the susceptible Boran cattle.

Earlier studies had proposed that immunosuppression in rodents was a result of the loss of antigen presenting cells. Mice infected with *T. rhodesiense* exhibited a progressive loss of antigen presenting macrophages from spleen cells, lymph nodes and peritoneal cavity (Bagasra, Schell and Le Frock 1981) and reduced expression of mannose, Fc-, and complement receptors (Grosskinsky *et al* 1983) which may lead to a reduction in antigen handling capabilities of antigen presenting cells. However, the

present understanding of immunosuppression in rodents is based on the regulation of MØ function and other effector cells such as $CD8^+$ T cells, than on the physical loss of cells. Spleen cells from *T. congolense* or *T. brucei* infected mice failed to respond to mitogenic stimulation *in vitro* and suppressed the ability of normal cells to respond to the same mitogens (Jayawardena and Waksman 1977; Mansfield *et al* 1981; Boroway *et al* 1990). It is possible that cells from infected animals were producing cytokines that inhibit cell proliferation but these studies did not investigate cytokine production. Later studies on cytokine production by mononuclear cells showed that IL-2 production by T cells was usually depressed in mice infected with *T. congolense* (Mitchell, Pearson and Gauldie 1986) and in *T. brucei* infection (Sileghem, Holmes and De Baetselier 1986). In addition, interferon gamma (IFN-γ) production increased in *T. brucei* and *T.cruzi* infected mice which appeared to be responsible for the dysfunction of T cells (Darji *et al* 1991). IFN-γ activated macrophages (MØ) are now at the centre of the proposed mechanism of immunosuppression in rodents. The theory is that trypanosomes release factors which activates $CD8^-$ T cells to release IFN-γ which activates MØ leading to the suppression of proliferative responses (Pinder 1984; Eardley and Jayawardena 1977). MØ inhibit proliferation via two proposed mechanisms; prostaglandin (PG) dependent and PG- independent mechanisms. In the PG-dependent mechanism, MØ interact with a trypanosomal fragment (70kD Mw) and secrete PG which actively inhibits IL-2 secretion by $CD4^-$ T cells. In the PG-independent mechanism, IFN-γ activated MØ release nitric oxide which then inhibit IL-2 receptor (IL-2R) expression on $CD4^-$ and $CD8^-$ T cells (Sileghem *et al* 1989c; Darji 1991). IL-2 promotes mononuclear cell proliferation, therefore any stimulus that inhibits its production or receptor expression will inhibit cell proliferation.

The role of IFN-γ in suppressing cell proliferation in rodents has been tested by culturing cells in the presence or absence of anti-IFN-γ antibodies. Results indicated that cells cultured in the presence of antibodies proliferated while those without antibodies to IFN-γ did not proliferate (Olsson *et al* 1991). In addition it has been

demonstrated that IFN-γ promotes *T. brucei* multiplication *in vitro*, and *in vivo* mice that were treated with anti-IFN-γ antibodies had lower parasitaemia and survived longer (Olsson *et al* 1991; Bakhiet *et al* 1996). The prostaglandin mechanism of suppression was tested by culturing mononuclear cells from mice infected with *T. brucei* in the presence or absence of indomethacin, a prostaglandin inhibitor. Cells with the inhibitor proliferated while those without it did not (Schleifer and Manfield 1993). The role of nitric oxide was also demonstrated by culturing mononuclear cells from *T. brucei* infected mice in the presence or absence of a nitric oxide synthetase inhibitor N-monomethy-L-arginine acetate (NMMA). Results showed that cells that were grown in the presence of the inhibitor proliferated while those grown without the inhibitor did not (Schleifer and Mansfield 1993; Sternberg and MacGuigan 1992). It was therefore, concluded that IFN-γ induced prostaglandin and nitric oxide were involved in the suppression of proliferative responses by mononuclear cells from *T. brucei* infected mice.

Although activated MØ and $CD8^-$ T cells from *T. congolense* infected cattle have been shown to suppress mononuclear cell proliferation *in vitro* (Taylor, Lutje and Mertens 1996; Flynn and Sileghem 1991) the mechanism seems to be different from that observed in rodents. The addition of indomethacin to inhibit prostaglandin synthesis had no effect on the ability of lymph node cells from cattle infected with *T. congolense* to suppress proliferative responses of responder cells from uninfected cattle (Flynn and Sileghem 1991; Taylor, Lutje and Mertens 1996). The addition of nitric oxide inhibitors to the cells had no effects on proliferative responses. In addition measurement of levels of nitric oxide and IL-10 in culture supernatants showed a decrease in concentration of nitric oxide while that of IL-10 increased as the infection progressed (Taylor, Lutje and Mertens 1996). These results demonstrate that prostaglandins and nitric oxide are not involved in the mechanism of suppressing proliferation of mononuclear cells from *T. congolense* infected cattle. Moreover, nitric oxide is down-regulated during the course of infection. Therefore, the mechanism in cattle may involve IL-10 a cytokine that is known to mediate suppression of MØ and effector cell function by inhibiting the expression of IFN-γ.

Suppression of non-specific cell-mediated responses

Non-specific suppression of cell-mediated immune responses has been observed in trypanosome infected rodents and ruminants. Spleen cells from *T. brucei* or *T. congolense* infected mice failed to respond *in vitro* to stimulation with either T cell mitogens such as concanavalin A (ConA) and phytohaemagglutinin (PHA) or to lipopolysaccharide (LPS) a B cell mitogen (Mansfield *et al* 1981; Pearson *et al* 1978 and 1979). Suppression of delayed type hypersensitivity reaction to purified protein derivative of tuberculin was observed in rabbits infected with *T. congolense* for 6 weeks (Mansfield and Wallace 1974). However, Freeman *et al* (1973) observed normal cell-mediated responses to *T. brucei* infection with suppression being observed only during the terminal stages of the infection probably due to differences in the virulence of trypanosomes used in the experiments. Investigations in cattle demonstrated that *T. vivax* infection in goats may be associated with more than 50% suppression of mitogen-induced lymphocyte function *in vitro*. Suppression was observed after 20 days of infection. Other studies showed no suppression of cellular responses e.g. there was no significant suppression of *in vitro* responses to mitogens for leukocytes from cattle infected with *T. congolense* or *T. vivax* (Masake and Morrison 1981; Sollod and Frank 1979; Morrison, Murray and Akol 1985). Strain differences in the ability to induce polyclonal proliferation may explain the differences in response. It seems most strains of trypanosomes induce polyclonal lymphocyte proliferation of un-stimulated cells *in vivo* leaving little or no naive cells to be stimulated by mitogens. Therefore, *in vivo* infection or antigens that induce non-specific cell mediated responses such as purified tuberculin protein derivative will be suppressed (Mansfield and Wallace 1974).

2.7 CONTROL OF ANIMAL TRYPANOSOMIASIS

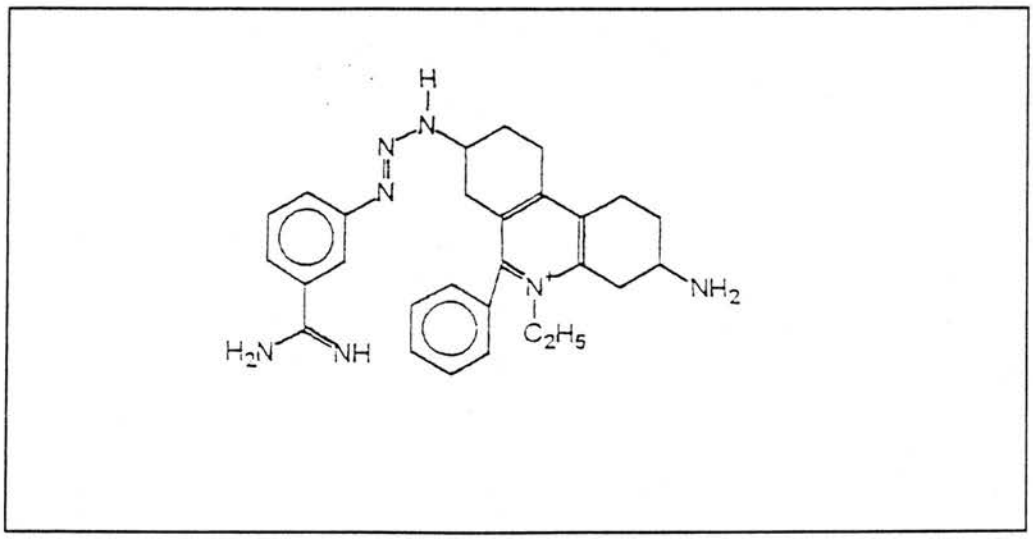
Control of trypanosomiasis is achieved by chemotherapy and chemoprophylaxis, vector control or eradication and breeding of trypanotolerant animals. However, integrated control programmes are usually employed.

2.7.1 Chemoprophylaxis

The use of suitable anti-trypanosomal drugs aimed at protecting animals from infection, forms a very important part of most integrated trypanosomiasis control programmes (Leach and Roberts 1981 review). Thus in the absence of a suitable vaccine, chemotherapy and chemoprophylaxis seem to be the most effective method available. (for reviews refer to Williamson 1970; Finelle 1976; Soulsby 1982; Kinabo 1993). Chemotherapy of trypanosomiasis caused by *T. congolense* in cattle depends on mainly three drugs; ISMM, homidium and diminazine. Diminazine and homidium are used as therapeutic agents and only ISMM is used for both therapeutic and prophylactic purposes (Whitelaw *et al* 1986; Kinabo 1993). ISMM is the sole agent used for prophylaxis against animal trypanosomiasis. It has greatly contributed to improving the productivity of livestock especially cattle reared in trypanosomiasis endemic areas and although it has been used since 1958 very little is known about its immunological effects, metabolic profile, teratogenic, mutagenic and carcinogenic effects. The hypothesis that it may improve immune responses to *T. congolense* is an attractive one because if verified it would prove a 'tried and tested' model of an immunomodulator that would be used in search of new prophylactic agents.

ISMM is a chloride salt chemically called 8- [(m- amidinophenylazo) amino] -3- amino -5- ethyl -6- phenylphenanthridium chloride hydrochloride. It is marketed under the trade names of (Samorin®, RMB Animal Health Ltd., Dagenham, U.K.) in Anglophone or (Trypamidium®, Rhone-Merieux, Lyon, France) in Francophone countries. It was first synthesised by coupling homidium with p-aminophenyldiazonium chloride (Wragg *et al* 1958) and subsequently the yield and purity was improved and the chemical structure elucidated (Berg 1960 and 1963). ISMM is a dark-brown powder which is cationic because it contains a quaternary nitrogen atom (Figure 2.3) and has a solubility of 6% in water at 20°C. It is less soluble in pure organic solvents and is labile at high temperature, and low and high pH conditions.

Figure 2.3 Chemical structure of ISMM



Hawking (1963) tested ISMM for activity against *T. congolense* and *T. b. rhodesiense in vitro* and found that it was ineffective up to concentrations of 100 000 ng/ml. However, Hill (1965) demonstrated that activity against *T. congolense in vitro* was less than that observed *in vivo*. These differences could be attributed to the possibility of potentiation by the immune system or metabolism to active metabolites *in vivo*. Activity against *T. congolense* was 10 - 20 times greater than that against *T. rhodesiense*. The difference is probably because *T. rhodesiense* usually resides in tissues where very little drug concentrations are found while *T. congolense* is usually found in the blood and lymphatics where high drug concentrations can be achieved. Further investigations on activity of ISMM have reviewed that the minimum effective concentration which killed *T. evansi* and *T. equiperdum* (MEC_{100}) in 24 hours of drug exposure was 1 000 - 4 000 ng/ml (Zhang, Giroud and Baltz 1991) and 1 - 300 ng/ml after 96 hours of exposure (Brun and Lun 1992). Culturing of trypanosomes, *in vitro* in the presence of 5 ng/ml ISMM for less than 10 minutes (Sutherland *et al* 1991b), 10 ng/ml for less than 24 hours (Kaminsky, Chuma and Wasike 1994) and 10 ng/ml for less than 48 hours (Gray and Peregrine 1993) could not completely eliminate the parasites or prevent infection. These results demonstrate that ISMM is trypanocidal at high but not at low concentrations. Nevertheless, ISMM serum or plasma concentrations during the protective period is usually less than 5 ng/ml, which

according to these results may have little trypanocidal effects. These observations lead to the hypothesis under present investigation that; ISMM provides protection against trypanosome infections by improving the responsiveness of the immune system. However, the difficulty has always been to provide evidence in support of this hypothesis.

Initial and successful therapeutic trials of ISMM on *T. congolense* in mice were performed by Wragg *et al* (1958), a few years before any *in vitro* assays were attempted. Subsequently a number of laboratory and field studies showed that the drug was active against *T. vivax*, *T. brucei*, and *T. evansi* infections in domestic animals (Kirkby 1963; Toure 1970; Arowolo and Ikede 1980; Ali 1985; Peregrine, Moloo and Whitelaw 1987). ISMM has also been shown to protect pigs from outbreaks of *T. simiae* infections for up to 5 months at doses of 12 - 35 mg/kg (Finelle 1973; Otaru and Nsengwa 1987). However, these doses are too toxic to be recommended for routine use (Kinabo, MacKellar and EcKersall 1991). The therapeutic dose of ISMM in animals other than pigs varies from 0.5 mg/kg in ruminants to10 mg/kg in mice.

Chemoprophylactic activity of ISMM against trypanosomes has been well documented, but the duration of protection after an intramuscular injection varies widely from as little as 2 weeks (Dolan *et al* 1992, Munstermann *et al* 1992) to 7 months (Weisenhutter *et al* 1970). However, sustained release devices containing ISMM have extended the mean period of protection up to 20 months at serum concentrations less than 1 ng/ml (Geerts *et al* 1997). The sustained release device was a cylindrical rod of 3 mm in diameter and 3 cm long consisting of a mixture of poly(D,L-Lactide) and 25% ISMM. The device was implanted subcutaneously in the shoulder region. The mean period of protection in the sustained release device group was 20 months compared to 5.7 months resulting from an intramuscular injection of a 2% ISMM solution at a dose of 0.5 mg/kg body weight. In the same study, no trypanolytic antibodies were detected during the protective period and based on that observation they concluded that only drug residues were responsible for the

protection. Nevertheless, many studies have shown that trypanolysis requires the presence of complement (Mansfield 1981). However, it has been demonstrated by other researchers that phagocytosis of trypanosomes in the liver alone is responsible for the clearance of more than half of the circulating trypanosomes in immunised but not naive mice (Holmes *et al* 1979; MacAskill *et al* 1980). This implies that *in vivo* opsonising antibodies are more relevant than trypanolytic antibodies, a view supported by the depletion of complement in trypanosome infected animals which means cellular immune responses may still be involved in animals protected by ISMM.

The primary mode of action that is currently considered to account for the molecular mechanism of anti-trypanosomal activity of ISMM is the blockade of nucleic acid synthesis through intercalation between DNA base pairs (Wagner 1971), inhibition of RNA polymerase (Richardson 1973), DNA polymerase (Marcus *et al* 1982) and incorporation of nucleic acid precursors into RNA and DNA (Lantz and Van Dyke 1972). Other proposed mechanisms include; inhibition of glycoprotein biosynthesis (Casero, Porter and Bernacki 1982), lipid metabolism (Dixon *et al* 1971), ATP metabolism (Frank-Henderson *et al* 1977), membrane transport (Girgis-Takla and James 1974) and selective cleavage of kinetoplast DNA (Shapiro and Englund 1990). However, effects on nucleic acid synthesis does not explain the selective toxicity of ISMM for trypanosomes *in vivo* since it also affects mammalian nucleic acids. However, effects on kinetoplast DNA, trypanothione which maintains intracellular redox balance (Fairlamb 1990) and glycosome organelles (Opperdoes 1985) might be more selective for trypanosomes but the precise role of each one is not known. Therefore, the true mechanism of action of ISMM on trypanosomes *in vivo* is yet unknown.

Experimental and field observations have demonstrated the existence of strains of trypanosomes resistant to ISMM (Schonefeld, Rottcher and Moloo 1987; Peregrine, and Whitelaw 1991; Codjia *et al* 1993). The actual mechanism of resistance is not known, but reports of reduced uptake of ISMM into trypanosomes have been postulated (Sutherland *et al* 1991a). However, the most interesting aspect of drug

resistance is the close association with the status of the host immune system. Repeated sub-curative drug treatment with cymerlarsan, berenil and ISMM was found to lead to the development of drug resistant trypanosomes only in immunosuppressed animals (Osman, Jennings and Holmes 1992). These results, once more underscore the importance of the immune system in trypanosomiasis chemoprophylaxis.

ISMM is normally administered via an intramuscular injection at 0.25 - 0.5 mg/kg for therapeutic and 0.5 - 1 mg/kg for prophylactic purposes. Although slow intravenous administration is effective for therapeutic purposes (Dowler, Schillinger and Connor 1989) it is associated with a reduced duration of protection (Sutherland *et al* 1991b). ISMM is poorly absorbed from subcutaneous sites and is not orally administered because of breakdown in acidic stomach and poor absorption (Philips *et al* 1967).

The kinetics of ISMM after an intramuscular administration vary widely. It is absorbed slowly and maximum plasma concentrations are achieved within 1 hour (Kinabo and MacKeller 1990). Twenty four hours after administration plasma level were 2170 ng/ml in goats (Braide and Eghianruwa 1980), below 10 ng/ml (Kinabo, McKeller and WcKersall 1991; Geerts *et al* 1997) and 40 - 50 ng/ml (Eisler *et al* 1994) in cattle. The concentration then falls rapidly to below 1 - 6 ng/ml by the end of one month after administration. However, despite plasma concentrations being below 1 ng/ml, most animals remained protected for up to 20 months (Geerts *et al* 1997). ISMM accumulates at concentrations higher than those found in plasma at the intramuscular injection site, liver, kidney, spleen, skeletal and cardiac muscles in that order (Kinabo and Bogan 1988). Little is known about metabolism but cumulative excretory data of [^{14}C] ISMM in faeces and urine of cattle given the drug intramuscularly (Kinabo and McKeller 1989; Kratzer *et al* 1989) suggested that the major route of excretion of unchanged ISMM and/or its putative metabolites is via the bile while excretion via urine is very low. Breakdown of ISMM to homidium has been demonstrated only in rats after an oral administration (Phillips *et al* 1967).

The therapeutic index of ISMM is very low and the maximum tolerated dose after intravenous administration were 2, 1.5, 1, and 0.05 mg/kg in dogs, cattle, camel and goats respectively in order of susceptibility (Schillinger, Moloo and Rottcher 1985). A dose of 1 mg/kg intravenously is lethal to 100% of goats, while 4 mg/kg is lethal to 50% of cattle (Robson 1962) and dogs (Phillips *et al* 1967). Signs of acute toxicity include; convulsions, flaccid paralysis of extremities and respiratory dysfunction probably due to blockade of neuromuscular transmission, direct stimulation of cholinoceptors and potentiation of histamine release (Phillips *et al* 1967; Arowolo and Eyre 1984). Acute toxicity is not common following an intramuscular injection because of slow absorption. However, the site is usually damaged by inflammation and fibrosis followed by coagulative necrosis. Leakage of drug to subcutaneous tissues may result in sloughing of the skin (Robson 1962; Kinabo and Bogan 1988b).

Due to the persistence of the drug in tissues of treated animals and its ability to intercalate with base pairs of nucleic acids, concerns have been raised on the safety of ISMM in meat destined for human consumption (Braide and Eghianruwa 1980; Kinabo and Bogan 1988d) and its mutagenicity potential (Lecointe *et al* 1981). Mutagenicity experiments demonstrated hepatic metabolism of ethidium (homidium) to reactive nitrogen species and although this has not been demonstrated for ISMM, the possibility of metabolic conversion to homidium (Phillips *et al* 1967) is what raises concerns. However, existing data on the stability of ISMM seem to indicates that the risk from the unchanged drug may be very low since it breaks down at low or high pH and at high temperatures, therefore can not withstand the process of cooking. Nevertheless, a maximum residual level for ISMM has not yet been recommended because of insufficient data particularly from carcinogenicity, teratogenicity and metabolic studies as well as short term oral administration studies (WHO 1989 and 1990).

2.7.2 Vector control

In isolated small areas with low or moderate tsetse density the control method of choice is eradication of the vector (Rommel 1983), through ground or aerial sprays of

insecticides with residual effects such as synthetic pyrethroids (MacLennan 1981; WHO 1986a). Insecticide impregnated traps or targets which requires the insecticide to be sprayed onto a trap or piece of cloth (target) coupled with a tsetse attractant, acetone and 1-octen-3-ol (Allsopp, Hall and Jone 1985) are also employed. Insecticides have also been sprayed on the body of animals (pour-ons) where the animal acts as a moving lethal target. There are experimental findings that ISMM is capable of eliminating the insect form of *T. vivax*, which may be of potential significance in the control of the disease in the field, particularly in relation to the operation of the sterile insect technique (Agu 1985). However, uninterrupted cycles in wildlife would still make this method less effective.

2.7.3 Breeding of Trypanotolerant animals

Trypanotolerance means reduced susceptibility to trypanosomiasis and denotes an inherited biological characteristic enabling animals to live, breed, grow and survive in a naturally infected environment without showing signs of disease after harbouring the trypanosomes (Murray, Morrison and Whitelaw 1982; Camus 1981). Trypanotolerance is found in local African breeds of cattle such as the N'dama (*Bos taurus*), sheep, goats and some pony types (Chabeuf 1983). Field studies by Njogu *et al* (1985) have demonstrated that even among Zebu (*Bos indicus*) cattle which are generally considered to be susceptible, some types of Borans *(Bos indicus)* can be more tolerant than others. However, breeding for trypanotolerance alone is not a sufficient control programme and in general, most trypanotolerant *Bos taurus* cattle have a low productivity compared to susceptible *Bos indicus*.

2.7.4 Induction of immunity by vaccination

The changing of the surface coat by blood stream forms (trypomastigotes) of trypanosomes has frustrated efforts in search of a vaccine (Crowe *et al* 1983; Vickerman and Barry 1982; Barry 1986). There are numerous examples of unsuccessful attempts to immunise animals with different strains of trypanosomes (Schilling 1935; Duxbury *et al* 1972; Wellde *et al* 1973). Immunisation with *T. rhodesiense* produced a strong resistance to challenge in cattle which lasted at least 8

months, but was waning by 14 months. The number of trypanosomes used was very large and success was only achieved with 6 weekly intravenous inoculations of about 10^{10} trypanosomes (Wellde *et al* 1973). Morrison *et al* (1982) using either single inoculum of 10^7 irradiated *T. brucei* or 200μg of variant specific surface antigens of *T. brucei* found that protection was conferred against challenge with 10^3 trypanosomes 14 days later. Also a group of mice which received 12 - 15 infective tsetse bites on two occasions 21 days apart, were resistant to infection when subjected to subsequent fly challenge with the same clone, while a second group of mice immunised and challenged by a clone from a different stock of *T. congolense* became infected (Nantulya, Doyle and Jenni 1980b). These results demonstrate that it is essentially easy to immunise animals against trypanosomiasis provided the challenge infection contains only the same variant antigen types as were used to immunise. However, antigenic variation has made it impossible for a field challenge to contain only one variant type to which animals would have already been exposed to. It is now established that a tsetse fly even when infected with a cloned population, after cyclical development inoculates a mixture of metacyclics into the mammalian host.

Trypanosomes remain uncoated for several weeks while developmental stages mature, during which period the fly ingests a blood meal every few days. If this blood contained antibodies against 'common' antigens of the uncoated trypanosomes, infection of the fly may be inhibited (Barry 1986). Nevertheless, this approach has been tried by immunising goats with freeze dried procyclic trypanosomes and then infecting them with *T. congolense*, *T. vivax*, and *T. brucei* (Murray, Morrison and Whitelaw 1982). When parasitaemia developed, tsetse flies were allowed to feed on the goats and it was subsequently found that *T. congolense* infection rates for these flies was significantly reduced as compared to tsetse flies fed on unimmunised goats. No difference was observed with *T. vivax* or *T. brucei*. However, were this approach successful, it would still have the practical reservation that unimpeded cyclical development would still occur in flies feeding on wild animals. However, traces of hope exist in the development of a vaccine based on the surface coat expressed by metacyclic forms in the tsetse fly since the overall composition of the metacyclic

surface coat is relatively constant although it may change with time (Nantulya, Musoke and Moloo 1986).

Murray and Morrison (1979) were able to induce an increased non-specific host defence activity (e.g. macrophage function) against *T. congolense* by using *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*. These agents were able to enhance the immune response to *T. congolense* infections in susceptible A/J mice and more resistant C57BL/6J mice, both showing reduced parasitaemias and increased survival times. The development of effective immunostimulants may provide attractive complementary tools for future control of trypanosomiasis, but so far nothing much has been done in this area.

2.7.5 Immune responses and chemotherapy in trypanosomiasis

The combined effects of chemotherapeutic agents and the immune system against trypanosome infections remain in its infancy. Experimental evidence has shown that the immune system plays a significant role in the effectiveness of difluorometlylornithine (DFMO) chemotherapy in African trypanosomiasis in mice (DeGee, MacCann and Mansfield 1983). Also repeated sub-curative drug treatment with cymerlarsan, berenil or ISMM was found to lead to the development of drug resistant trypanosomes only in immunosuppressed animals (Osman, Jennings and Holmes 1992). These results suggest that the state of the host immune system may play an important role in the development of drug resistance. However, the role played by the immune system on the efficacy of isometamidium, the sole prophylactic drug against livestock trypanosomiasis is not well understood. The relationship between immunocompetence and prophylaxis is of great clinical importance since it is known that trypanosome infections and other stress factors are associated with immunosuppression.

Cattle infected with *T. congolense* and treated with Berenil developed characteristic delayed type hypersensitivity skin reactions when inoculated intradermaly with ultrasonicated *T. congolense* antigen. PBLs from these animals proliferated *in vitro*

when collected before chancre development or after drug treatment but not during the period of active infection (Emery and Moloo 1980). Immunisation of goats with tsetse transmitted *T. congolense* and drug treatment after chancre development on day 13 post infection resulted in protection against homologous challenge. On the other hand immunisation by intravenous inoculation of metacyclics or treatment of tsetse infected goats before chancre development failed to produce protection (Taiwo *et al* 1990).

Morrison, Murray and Akol (1985) showed that cattle experimentally infected with trypanosomes could be made immune to homologous challenge by a brief period of infection followed by trypanocidal drug treatment. Cunningham (1968) and Wilson (1971) obtained similar results but Wellde *et al* (1981) failed to induce protection in cattle infected with *T. congolense* and treated with Berenil at various intervals after infection, then challenged with the parent stock 28 - 128 weeks after treatment.

Protective immunity induced against trypanosome infection and drug treatment is said to be specific for the surface coats that develop during the course of infection (Nantulya *et al* 1980b; Akol and Murray 1985). Rabbits immunised by cyclical infection and treatment with homidium chloride 7 days after development of chancre showed immunity lasting about 10 months (Luckins and Gray 1983). Nevertheless, animals that recover from the infection on their own, also develop protective immunity to subsequent homologous challenge. Cattle that self cured from a primary *T. congolense* infection after 30 - 61 weeks became fully immune to the same stock 25 - 54 weeks after the parasitaemia had disappeared (Wellde *et al* 1981).

Serum IgM levels increase many times during infection and persist for quite sometime even after drug treatment (Luckins and Gray 1979; Boid *et al* 1980). No trypanolytic antibodies have been detected in prophylactically treated cattle infected with *T. congolense* six months later (Whitelaw *et al* 1986; Peregrine *et al* 1988). However, there is no existing information on the effect of drugs on T cell immune responses in prophylactically treated animals. In addition, these studies could not rule out the existence of opsonizing antibodies which are important in phagocytosis.

CHAPTER 3

CHAPTER 3
MATERIALS AND METHODS

Working solutions and a list of several reagents used in all experiments and their suppliers is provided in appendix I.

3.1 TRYPANOSOMES

Three isolates of *T. congolense* (TREU 1476 and TREU 1881) that had been adapted for *in vitro* axenic culture conditions were all stored in liquid nitrogen at the Centre for Tropical Veterinary Medicine. TREU 1476 was a blood stream form while both insect and blood stream forms of TREU 1881 were used.

3.2 EXPERIMENTAL ANIMALS

Scottish black face male sheep approximately two and a half years and average body weight of 41kg were obtained from Moredun Pentlands Buildings and maintained on a normal diet and management at the Centre for Tropical Veterinary Medicine animal quarters. Twelve week old female T.O. outbred mice were supplied by A.Tuck and Son Ltd, Beeches Road, Battlebridge, Essex, S11 8TJ, U.K., while C57 BL/6 and BALB/c inbred strains of mice were from B&K Universal, Grimston, Aldborough, Hull, HU11 4QE, U.K.

3.3 EXPERIMENTAL DESIGN

Three groups of sheep were used for trypanosome infection experiments (Table 3.1). The prophylactic group was injected with 1 mg/kg ISMM intramuscularly. Plasma was collected daily for four days, then 7, 14 and 21 days after treatment and stored at -20°C till required for drug concentration determination. Blood in EDTA was collected weekly for the determination of total RBC, total WBC, differential WBC, PBMC phenotypes, proliferation and IFN-γ production *in vitro*. Four and half months after ISMM treatment of the prophylactic group all three groups were inoculated with 10^6 metacyctics of *T. congolense* isolate TREU 1881 and rectal temperature was recorded daily till termination of experiment 35 days post infection. Plasma collected 35 days post infection was stored at -20°C and later used to determine trypanosome specific IgG antibodies.

Table 3.1 Experimental design for ISMM and sheep trypanosome infection

EXPERIMENTAL GROUP	N	PARAMETERS	
		PRE-INFECTION	**POST-INFECTION**
Control	4	IFNγ, phenotypes, *in vitro* proliferation,	Clinical, parasitaemia, IFN-γ, phenotypes, *in vitro* proliferation, IgG antibodies
Prophylactic	4	ISMM plasma conc, IFN-γ, phenotypes, *in vitro* proliferation,	Clinical, parasitaemia, IFN-γ, phenotypes, *in vitro* proliferation, IgG antibodies
Treated	4	IFNγ, phenotypes, *in vitro* proliferation,	Clinical, parasitaemia, IFN-γ, phenotypes, *in vitro* proliferation, IgG antibodies

N = number of sheep per group

The BCG-ISMM group (Table 3.2), was first injected with 1 mg/kg ISMM and 14 days later both groups were inoculated with 5 human doses of BCG vaccine. Blood in EDTA was collected weekly from 14 days before BCG inoculation to 35 days after inoculation for the determination of PBMC phenotypes and estimation of proliferation *in vivo*. When the experiment was terminated 35 days after inoculation at which point, two 0.1 ml of PPD was injected intradermaly to determined delayed type hypersensitivity reaction.

Table 3.2 Experimental design for ISMM and sheep BCG Vaccination

EXPERIMENTAL GROUP	N	PARAMETERS	
		PRE-VACCINATION	**POST-VACCINATION**
BCG-Control	3	Phenotypes, *in vivo* proliferation,	Phenotypes, *in vivo* proliferation, PPD skin test
BCG-ISMM	3	Phenotypes, *in vivo* proliferation	Phenotypes, *in vivo* proliferation, PPD skin test

N = number of sheep per group

Four groups consisting of 20 mice were used for determining the efficacy of ISMM in mice (Table 3.3). Group one was used as control, group two was given 0.02 mg hydrocortisone daily for four consecutive days, group three received a single prophylactic dose of 1 mg/kg ISMM. Group four was given a single prophylactic dose of 1 mg/kg ISMM and 0.02 mg hydrocortisone daily for four consecutive days. Eleven days post ISMM treatment all groups were inoculated with 10^6 trypanosomes

intraperitoneally. Thereafter they were examined for parasitaemia, pre-patent period and survival time.

Table 3.3 Experimental design for efficacy of ISMM in mice

EXPERIMENTAL GROUP	N	PARAMETERS POST-INFECTION
Control	5	Parasitaemia, pre-patent period and survival time
Hydroco.	5	Parasitaemia, pre-patent period and survival time
ISMM	5	Parasitaemia, pre-patent period and survival time
ISMM +Hydroco.	5	Parasitaemia, pre-patent period and survival time

N = number of mice per group
Hydroco. = Hydrocortisone

Table 3.4 shows the chronology of injecting ISMM to mice for determining its effects on IL-12 and IFN-γ production. Three mice were injected with ISMM 21, 14, and 7 days before *in vitro* culture of splenic cells. All mice including 3 for the untreated control group were killed on the same day and spleens aseptically removed for the separation of splenic cells.

Table 3.4 Experimental design for effects of ISMM on IL-12 and IFNγ production in mice

EXPERIMENTAL GROUP	N	PARAMETERS
Control	3	IL-12 and IFN-γ production *in vitro*
Day 7	3	IL-12 and IFN-γ production *in vitro*
Day 14	3	IL-12 and IFN-γ production *in vitro*
Day 21	3	IL-12 and IFN-γ production *in vitro*

N = number of mice per group

3.4 Determination of ISMM plasma profile

Plasma was collected from the four prophylactically treated sheep at certain intervals post treatment for measuring ISMM levels. The method used is a competitive ELISA as described by Eisler *et al* (1994). The principal of the assay is that free drug in plasma competes with ISMM-horseradish peroxidase conjugate for binding to anti-ISMM antibodies bound to the plate. The intensity of colour produced is inversely proportional to the amount of free drug present in the sample. Therefore, a high optical density means drug concentration is low and vice versa. Rabbit anti-ISMM antibodies were diluted 1.8000 in carbonate/bicarbonate coating buffer. 100 µl were

dispensed into 96 well plates and incubated overnight at 4°C. Plates were stored at -20°C in situ for not more than 3 months. Prior to use plates were thawed and brought to room temperature. The following 1:3 serial dilutions of ISMM standards were prepared: 500, 166.7, 55.55, 18.51, 6.17, 2.05, 0.686, 0.229, 0.076 and 0.0225 ng/ml. ISMM-horseradish peroxidase conjugate was diluted 1: 128 000 in PBST. Each sample/standard was diluted 1:10 in the diluted ISMM-horseradish peroxidase conjugate. Thawed plates were then washed five times and 100 µl of sample/standard plus ISMM-horseradish peroxidase were added to anti-ISMM coated wells in duplicate. 100 µl of PBST were added to a blanking well. Plates were then incubated at 4°C overnight. A 1:1 dilution of substrate/TMB chromogen was prepared and warmed-up to 37°C. Plates were again washed five times, after which 100 µl of substrate/chromogen were added and incubated at 37°C for 10 minutes. Then 100 µl of 2M ortho-phosphoric acid were added to stop the reaction. The plate was vortex mixed for 2 minutes before reading absorbency at 450 nm on a Multiscan ELISA plate reader. A plot of absorbency (Y-axis) against standard concentration (X-axis in log units) was made. Sample concentrations were obtained by extrapolation from the standard graph.

3.5 *In vitro* efficacy of ISMM on *T. congolense*

T. congolense isolate TREU1467 and TREU1881 were cultured axenically according to the method of Ross and Taylor (1990) at an initial concentration of 5×10^5 trypanosomes/ml in 96 well culture plates in a humidified 5% carbon dioxide incubator at 33.5°C. ISMM was incorporated in cultures at an increasing concentration of 0, 1, 10, 100, and 1000 ng/ml. Promega Cell Titer96® Proliferation assay was used to determine the proportion of viable cells present in cultures. After a 48 hour incubation period, 15 µl of Promega dye solution were added directly into culture wells. Incubation was continued for 4 hours after which 100 µl of solubilisation/stop solution were added to all wells. Plates were left in a humid chamber overnight to allow blue formazan crystals to solubilise, thereafter absorbency was read on a Multiscan ELISA plate reader at 570 nm. The assay is non-radioactive and is based on a viable cellular enzymatic conversion of a tetrazolium salt into a formazan product that is easily detected using an ELISA plate reader at 570 nm.

Colour intensity is proportional to the number of viable cells present. Inhibition of trypanosome growth was calculated as a percentage of the control cultures subtracted from 100 since assay readings corresponded to the number of viable not dead cells present.

3.6 Clinical, haematological and parasitological examinations

All animals inoculated with trypanosomes were observed frequently for any clinical signs. Parasite detection and packed cell volume (PCV), were performed using the heamatocrit method where capillary tubes containing EDTA blood were centrifuged at 10 000 g (Biofuge). After recording the PCV, the buffy coat was examined for parasites under the microscope. Total red and white cell counts were performed using a Coulter counter. Differential white cell counts were performed by fixing thin blood smears with methanol for 1 minute followed by staining in 10% Giemsa stain for 10 minutes. Slides were examined under a microscope and neutrophils, eosinophils, monocytes and lymphocytes were scored out of a total of 200 cells counted. Rectal temperatures were taken every morning after infection using a digital thermometer.

3.7 Preparation of peripheral blood mononuclear cells (PBMC)

Approximately 14 ml of venous blood was collected from each sheep using EDTA as an anticoagulant. Samples were centrifuged at 1000 g for 20 minutes at 4°C. The buffy coat was then suspended in 9 ml of PBS containing 20 U/ml heparin (wash buffer). This was slowly layered on top of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 1000 g for 35 minutes. PBMC were removed from the interface and washed twice in wash buffer at 350 g for five minutes. Cells were re-suspended in 5 ml of culture media (RPMI 1640 supplemented with, 7% foetal calf serum and 100 U/ml penicillin plus 100 mg/ml streptomycin). They were counted on a heamocytometer and cultured at a density of 5×10^6/ml in 96 well culture plates in a volume of 200 µl media at 37°C for the required period of time.

3.8 Cell phenotype analysis

For each sample, 200 µl of cell suspension prepared above were put in FACS tubes labelled; blank, CD4, CD5, CD8, γδ and B cell. Cells were washed once with ice cold

wash buffer by centrifuging at 350 g for 5 minutes. Then 25 µl of wash buffer were added to blank tubes and 25 µl of each antibodies (undiluted) to appropriate test tubes and incubated at 4°C for 30 minutes. Cells were then washed twice and 25 µl of 1:1000 FITC-conjugated antimouse-isotype were added to appropriate tubes including blanks and incubated for 30 minutes at 4°C in the dark. After that cells were washed once and re-suspended in 200 µl of wash buffer and 200 µl of 1% paraformaldehyde and kept at 4°C till analysis on the flow cytometer. The FACSAN cell analyser was used for analysis. 10 000 cells analysed and data calculated in histogram mode. Different cell populations were detected using Forward (FSC) and Side (SSC) Scatter parameters (in linear amplification mode). Fluorescent light emission were detected in logarithm amplification mode. Cell phenotype proportions obtained from the FASCAN for trypanosome experiments were converted to absolute number of cells per millilitre of blood by first calculating absolute lymphocyte numbers by multiplying total white cell counts by the percentage of lymphocytes in blood. Then, multiplying the percentage of each phenotype by the absolute lymphocyte count yields the absolute phenotype count.

3.9 Determination of PBMC proliferation *in vitro*

Peripheral blood mononuclear cells were prepared from blood in EDTA as described in section 3.6. Each sample was divided into three for culture with: $2.5x10^5$/ml live *T. congolense*, 6.25 µg/ml ConA, and normal media. Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere for 72 hours. Proliferation of cells was estimated using the Promega Cell Titer96® Proliferation assay and verified by flow cytometry.

3.9.1 Cell proliferation using Promega Cell Titer96® Proliferation assay

The Promega assay is non-radioactive and is based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected using an ELISA plate reader at 570 nm. It has an advantage over other assays, of being able to detect an increase in cell numbers as well as activation-induced programmed cell death. However, the assay is not as sensitive as the radio-labelled thymidine uptake assay, but has the advantage of being able to detect activation-induced programmed cell death, which the radio-labelled thymidine uptake assay cannot detect.

PBMCs were cultured in 96 well plates in 200 µl of media (RPMI 1460 containing 7% foetal calf serum) at a density of 5 x 10^5 cells per well with or without 2.5 x 10^5/ml trypanosomes or 6.25 µg/ml ConA. After the 72 hour incubation period, 30 µl of dye solution were added to each well and incubation continued for a further 4 hours at 37°C in a humidified 5% CO_2 atmosphere. Thereafter, 100 µl of solubization/stop solution were added to all wells. Plates were left to stand overnight (18 - 24 hrs) in a sealed humidified container at room temperature to completely solubilize the blue formazan crystals. Absorbance was read at 570 nm wavelength using a Multiscan ELISA reader. Colour intensity is proportional to the number of viable cells present. The stimulation index was obtained by dividing the test absorbance reading by that of media only. An index of 1 indicates that the number of viable cells in the test well is the same as that in the media only (blank) well. A value above 1 indicates that there is an increase in cell numbers (proliferation) while that below 1 means that there is accelerated cell death in test cultures.

3.9.2 Cell proliferation using Flow Cytometry

The use of flow cytometry is a novel approach which is non-radioactive and is more sensitive than the Promega Cell Titer96® Proliferation assay. The method also has a further advantage of being able to detect proliferation *in vivo* by analysing freshly prepared WBCs or PBMCs. But like the radio-labelled thymidine uptake assay, it is not capable of detecting activation induced programmed cell death. Determination of cell proliferation using Flow Cytometry is based on the principle that cells increase in size (blast) when they are activated before they divide. The difference in size between normal un-stimulated cells and blast cells can be detected by the flow cytometer. The percentage of blast cells is directly proportional to the stimulation of cells to proliferate. PBMCs from cultures or prepared straight from the animal were suspended in 400 µl of PBS in flow cytometry tubes. 10 000 mononuclear cells were acquired through a dot plot display without the need for antibody staining and stored on computer for further analysis. Stored events were analysed on a dot plot display by setting two gates, one for all mononuclear cells and the other for blast cells only. For *in vitro* assays, the stimulation index was obtained by dividing the percentage of

lymphoblasts in test sample by that of the media only. An index of 1 indicates that the number of viable cells in the test well is the same as that in the media only (blank) well. *In vivo*, the percentage of lymphoblasts increases above pre-infection levels. The reference normal level of lymphoblasts depends on the gating of the two cell populations but does not affect the stimulation index obtained since it is a ratio.

3.10 IFN-γ determination assays

3.10.1 Sheep IFN-γ assay

PBMC preparation and culture plate set-ups were carried out as described in section 7.2.1 above. After a 48 hour incubation period culture supernatants were collected and stored in vials at -70°C. Sheep IFN-γ levels in supernatants were determined using the Bovine γ interferon Test. The test recognises bovine, ovine and caprine IFN-γ. It is a solid phase sandwich enzyme immunoassay designed to detect biologically active IFN-γ in plasma. The use of the test in ovine and caprine IFN-γ detection has been reported to be effective in demonstrating cell mediated immunity to various experimental antigens, T cell epitopes and adjuvant formulations in these species. The test will detect less than 50 pg/ml of recombinant IFN-γ or less than 1 unit of "natural" IFN-γ.

Test samples were assayed in duplicate in adjacent wells while positive and negative controls were assayed in triplicate serial wells. 50 µl of diluent were added to the required number of wells followed by 50 µl of test and control samples. Control samples were added last to each plate, mixed well, and incubated at room temperature for 60 minutes. Plates were washed five times and 100 µl of freshly prepared conjugate reagent added to all wells, then left to incubate at room temperature for 60 minutes. Thereafter, plates were once again washed 5 times and 100 µl of freshly prepared enzyme substrate solution added. Plates were incubated for 30 minutes at room temperature, after which 50 µl of stop solution were added to all wells. After mixing by gentle agitation, absorbance was read at 450 nm within 20 minutes of terminating the reaction blanking on air. Control results were examined before sample results could be interpreted. Firstly, the mean absorbance of the negative and positive controls were calculated. Results were acceptable if the ratio

of positive to negative mean controls was greater than 7.5. The cut-off point was taken to be the mean of the negative control plus two times its standard deviation.

3.10.2 Mouse IFN-γ assay

All mice shown in Table 3.4 were killed and spleens were aseptically removed, and mashed on sterile stainless steel mesh to separate mononuclear cells from connective tissue. Splenocytes were then suspended in 1 ml of culture media, counted and their concentration adjusted to $5x10^6$/ml. 100 µl of each sample were added to 96 well plates followed by 100 µl of media or 100µl of two times the desired final concentration of LPS (i.e. 2 µg/ml) or trypanosomes (i.e. $5x10^5$/ml) in culture media. Plates were then incubated for 24 hours at 37°C in a 5% CO_2 humidified incubator, after which supernatants were collected and used to assay IFN-γ and IL-12 immediately.

The assay used was the InterTest-γ™ Mouse IFN-γ ELISA which is a solid-phase ELISA employing the multiple antibody sandwich principle. The assay can detect upto 5 pg/ml mIFN-γ. Samples were assayed in duplicate and negative and positive (540 pg/ml) controls in quadruplicates. First, 100 µl of control and test samples were added to 96-well microtitre plate (pre-coated with anti-mIFN-γ antibody) and incubated for 1 hour at 37°C. Plates were washed 4 times and 100 µl of biotinylated anti-mouse-IFN-γ added to each well and incubated for 1 hour at 37°C. Plates were again washed 4 times and 100 µl of horseradish peroxidase - conjugated streptavidin were added to all wells and incubated for 30 minutes at 37°C. After washing 4 times 100 µl of TMB substrate reagent were added and incubated for 15 minutes at room temperature. Colour development was stopped by adding 100 µl of stop solution to every well. The ELISA reader was blanked on air and absorbance read at 450nm. Results were acceptable if the ratio of positive to negative mean controls was greater than 7.5. The cut-off point was taken to be the mean of the negative control plus two times its standard deviation.

3.11 Mouse IL-12 determination assay

Total IL-12 and IL-12p70 in supernatants from cultures were assayed as described below.

3.11.1 Total Mouse IL-12 assay

InterTest™-12X Total Mouse IL-12 ELISA Kit is a solid-phase ELISA employing the multiple antibody sandwich principle. A 96-well microtitre (pre-coated with anti-mouse IL-12p40) was used to capture mouse IL-12 from control and test samples. Three secreted forms of IL-12 have been identified and this assay will detect all the three forms: p70 heterodimer, $p40_2$ homodimer and p40 monomer. All samples were run in duplicate while negative and positive (540 pg/ml) controls were run in quadruplicates. 50 µl of assay diluent and 50 µl of samples were added to wells, while 100 µl of controls were added without further dilutions followed by incubation at room temperature for 30 minutes. Plates were washed 4 times and 100 µl of anti-mouse IL-12 biotinylated antibody added to all wells, then incubated at room temperature for 30 minutes. After another similar wash 100 µl of horseradish peroxidase-conjugated streptoavidin were added and incubated at room temperature for 15 minutes. At the end of this incubation plates were washed again 4 times and 100 µl of substrate added and incubated for 10 minutes at room temperature. Reaction was stopped by the addition of 100 µl of stop solution. After mixing well, absorbance was read at 450 nm. Results were acceptable if the ratio of positive to negative mean controls was greater than 7.5. The cut-off point was taken to be the mean of the negative control plus two times its standard deviation.

3.11.2 Mouse IL-12p70 assay

InterTest™-12X Mouse IL-12p70 ELISA Kit is also a solid-phase ELISA employing the multiple antibody sandwich principle. A 96-well microtitre (pre-coated with anti-mouse IL-12p35) was used to capture mouse IL-12p70 from control and test samples. All samples were run in duplicate while negative and positive (540 pg/ml) controls were run in quadruplicates. 50 µl of assay diluent and 50 µl of samples were added to wells, while 100 µl of controls were added without further dilutions followed by incubation at room temperature for 2 hours while shaking at 150g. Plates

were washed 4 times and 100 µl of anti-mouse IL-12 biotinylated antibody added to all wells, then incubated at room temperature for 1 hour while shaking at 150g. After another similar wash 100 µl of horseradish peroxidase-conjugated streptoavidin were added and incubated at room temperature for 15 minutes with shaking at 150g. At the end of this incubation plates were washed again 4 times and 100 µl of substrate added and incubated for 20 minutes at room temperature without shaking. Reaction was stopped by the addition of 100 µl of stop solution. After mixing well, absorbance was read at 450 nm. Results were acceptable if the ratio of positive to negative mean controls was greater than 7.5. The cut-off point was taken to be the mean of the negative control plus two times its standard deviation.

3.12 Trypanosome specific plasma IgG antibody titration

Antigens from *T. congolense* isolate number TREU 1881 were prepared in PBS by three cycles of freeze thaw at -20°C. The lysate was centrifuged at 3000 rpm. The supernatant was collected and the protein concentration estimated using a modified method of Lowry *et al* (1951). Antigen was diluted in carbonate/bicarbonate coating buffer pH 9.6 at a final protein concentration of 1 µg/ml. 100 µl of diluted antigen were added to all wells of a 96 well Immulon® 4 plate. Plates were incubated overnight at 4°C. A serial dilution of negative and test sera was performed in a 96 well culture plate in duplicate columns. Final serum dilutions were; 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, 1:8192, 1:16384, 1:32768, and 1:65536. Antigen coated plate were washed 4 times with 0.05% tween 20 in PBS (wash buffer). Then 100 µl of serial diluted sera was added to coated plates and incubated at 37°C for 30 minutes. After incubation plates were washed four times. Then, 100 µl of 1:30000 anti-bovine-IgG-horseradish peroxidase conjugate was added to all wells and incubated for 30 minutes at 37°C. Again plates were washed four times and 100 µl of substrate/chromogen (H_2O_2/TMB) were added and plates incubated for 15 minutes at 37°C. After that 100 µl of 1M ortho-phoshoric acid (H_3PO_4) were added and optical density read at 450 nm. The antibody titre was the highest dilution that gave a positive result after subtracting readings for the negative serum.

3.13 Statistical analysis of results

Statistical analysis of results was done in two ways. The first was within group analysis where pre-infection results were compared with post infection results. For some results, the second analysis was carried out between groups, where comparisons were made between the control and the prophylactic or treated groups during the period during the post infection or post treatment periods. Some of the results were, normally distributed while others were not, therefore all results were analysed non-parametrically using the Mann-Whitney test (GraphPAD Instat, GraphPAD Software, version 1.13, 1990).

The magnitude of fever (estimated from rectal temperature) was compared by performing statistical analysis on the area under the temperature time curve using the trapezoidal equation rule (Hail 1958) for all animals. Approximation of the area under the graph was done in four steps: (1) data of temperature and time was plotted on a 5 mm square grid such that each unit of time was 5 mm and each decimal unit of temperature was also 5 mm. (2) the plot was then divided into 5mm wide panels on a baseline at 40°C. (3) the number of grid points between 40°C and each observed temperature were counted. (4) the approximate area under the temperature curve was calculated from the trapezoidal rule equation as follows:- $Area = (h)(w)[\{y_o+y_n/2\}+y_1+y_2+ \ldots\ldots y_{n-1}]$, where h is panel height, w is panel width, and y_o, y_1 ,y_2 .....y_n are panel numbers. Tables of representative statistical analyses are provided in Appendix VII.

CHAPTER 4

CHAPTER 4
PARASITOLOGICAL, CLINICAL AND HAEMATOLOGICAL RESPONSES

4.1 INTRODUCTION

Haematological changes in white and red cells have been well documented as major pathophysiological feature of trypanosomiasis in domestic livestock (Valli and Mills 1980: Ellis *et al* 1987). Infection with *T. congolense* resulted in lymphocytopaenia followed by lymphocytosis and anaemia after the onset of parasitaemia (Valli, Forsberg and Mills 1979; Ellis *et al* 1987; Williams *et al* 1991). Fever usually fluctuates with waves of parasitaemia when the disease enters the chronic stage (Williams *et al* 1991; Onah 1990). Similar haematological changes were observed in sheep infected with *T. congolense* (Mwangi 1991) and *T. evansi* (Onah 1990).

Parasitological examination, rectal temperature and haematological analysis constitute reliable methods of monitoring the pathology of the infection. Anaemia is the main pathological lesion and is clinically determined by the packed cell volume. Severe loss of red blood cells results in a decrease in the packed cell volume. In addition to disease monitoring, haematological parameters mark the starting point for studying white blood cell subsets in the peripheral blood. Therefore, these parameters are discussed in this chapter.

4.2 MATERIALS AND METHODS

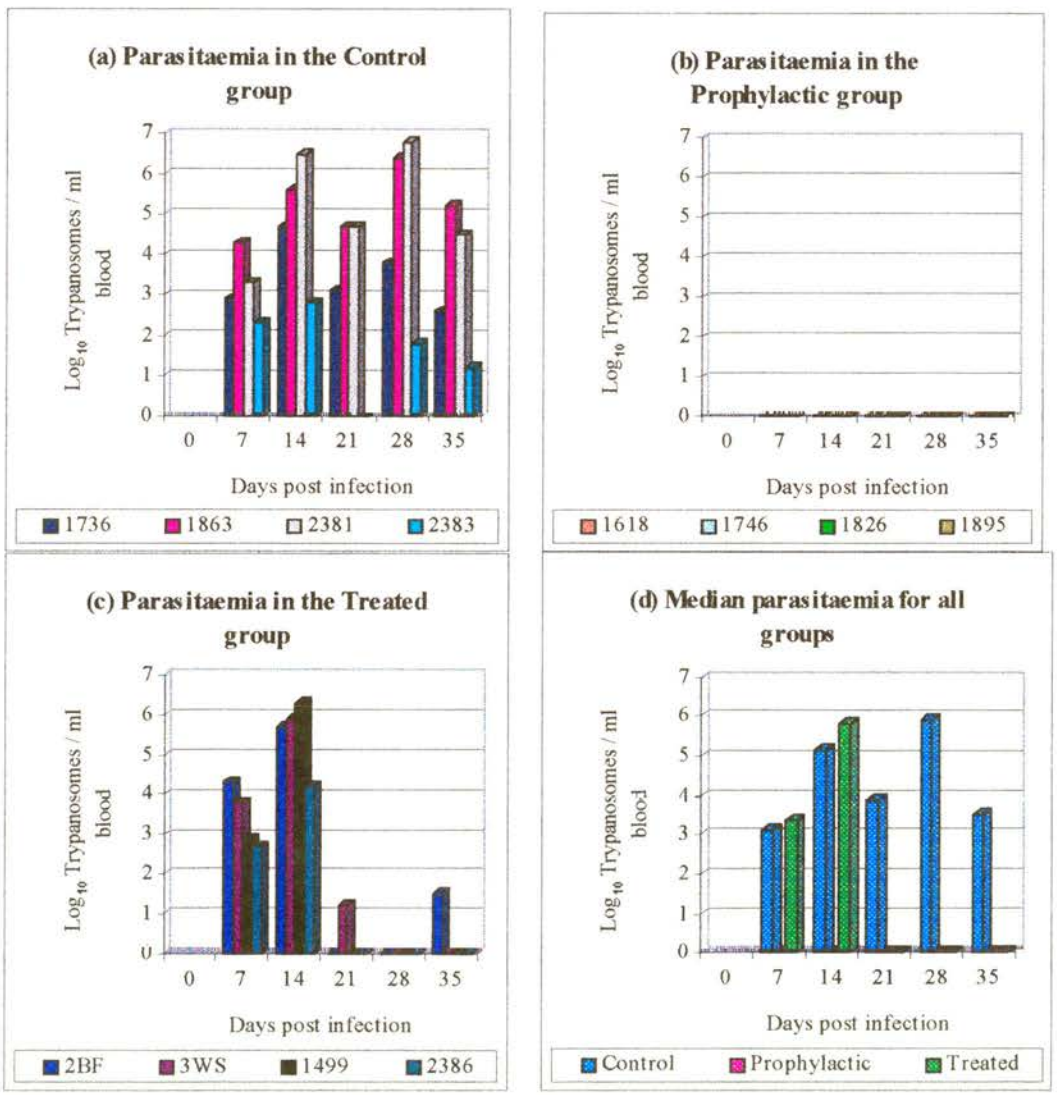
Parasitaemia, rectal temperature, total and differential cell counts were determined as described in Chapter 3, section 3.6. Parasitaemia and cell counts were determined once a week while PCV was performed twice a week and rectal temperature was taken every morning throughout the study period.

4.3 RESULTS

4.3.1 Parasitaemia

Parasitaemia was estimated by counting parasites from the buffy coat on a heamocytometer. Results presented in Figure 4.1 indicate that there were no parasites detected in the prophylactic group using the haematocrit method throughout the infection period. Animals in the control and treated groups were all parasitaemic 7 day post infection. The control group remained parasitaemic throughout the study period. Sheep 1863 and 2381 from the control group were jaundiced by day 28 post infection. Parasites were subsequently cleared from the treated group, except sheep 2BF which relapsed by 35 days post infection.

Figure 4.1 Parasitaemia after inoculation with *T. congolense* on day 0.

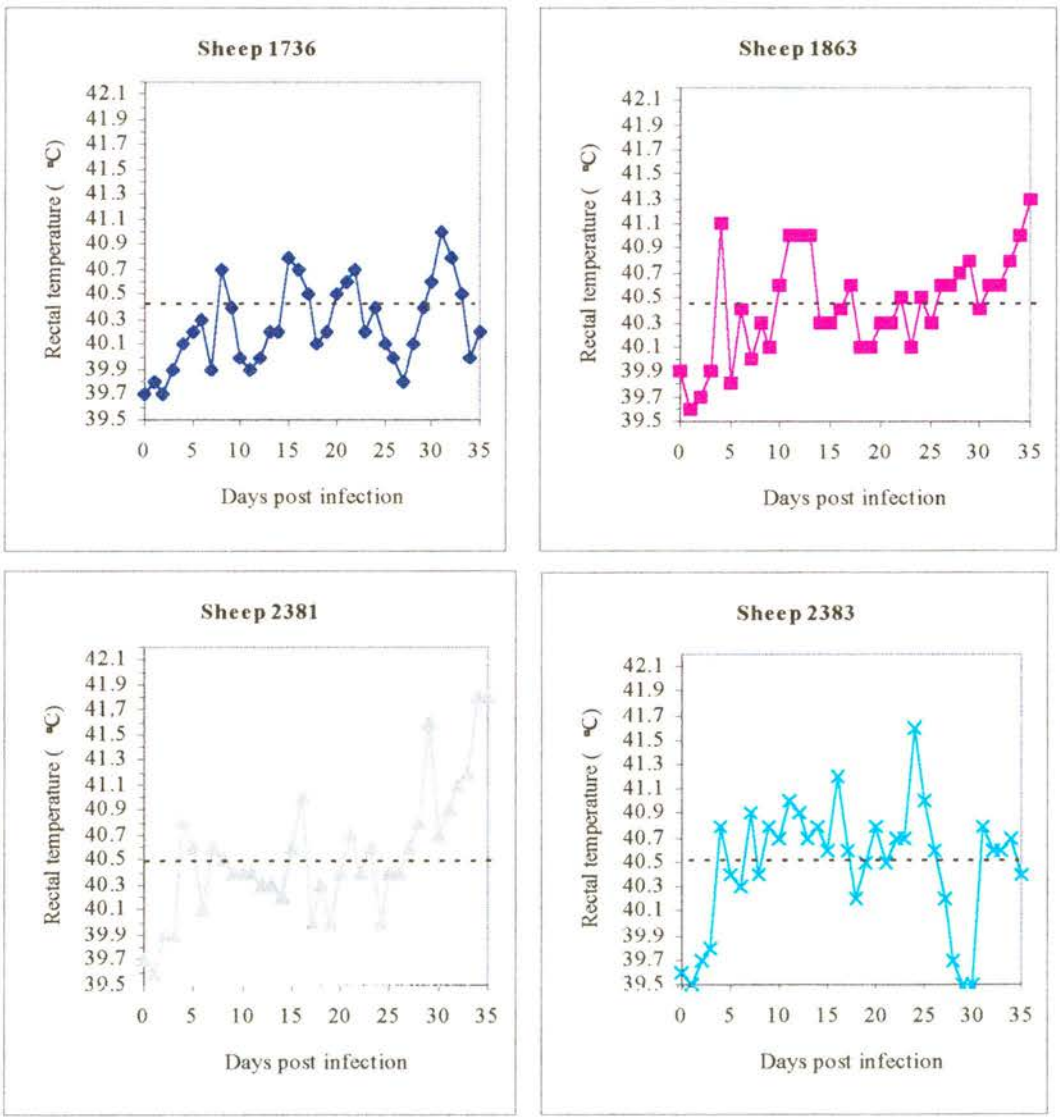


4.3.2 Determination of fever

The rectal temperature was taken every morning and used to monitor the progression of fever. Rectal temperature of 40.5°C was taken as the critical value. Recordings above the critical value were indicative of a febrile state. In order to make a quantitative comparison of fever, the area under the temperature time curve was approximated in four steps: (1) data of temperature and time was plotted on a 5 mm square grid such that each unit of time was 5 mm and each decimal unit of temperature was also 5 mm. (2) the plot was then divided into 5 mm wide panels on a baseline at 40°C. (3) the number of grid points between 40°C and each observed temperature were counted. (4) the approximate area under the temperature curve was calculated from the trapezoid rule equation described in Chapter 3, section 3.13. The area obtained was proportional to the magnitude of fever.

Results indicate that the control group showed waves of fluctuating fever throughout the study period (Figure 4.2; Table A1(a)-Appendix II). The onset of fever ranged from 4 to 9 days post infection and the magnitude was highest in sheep 2381 and lowest in sheep 1736. The area under the curve being; 5575 mm^2 for sheep 2381, 5150 mm^2 for sheep 2383, 4500 mm^2 for sheep 1863, 3050 mm^2 for sheep 1736 and the median value was 4825 mm^2.

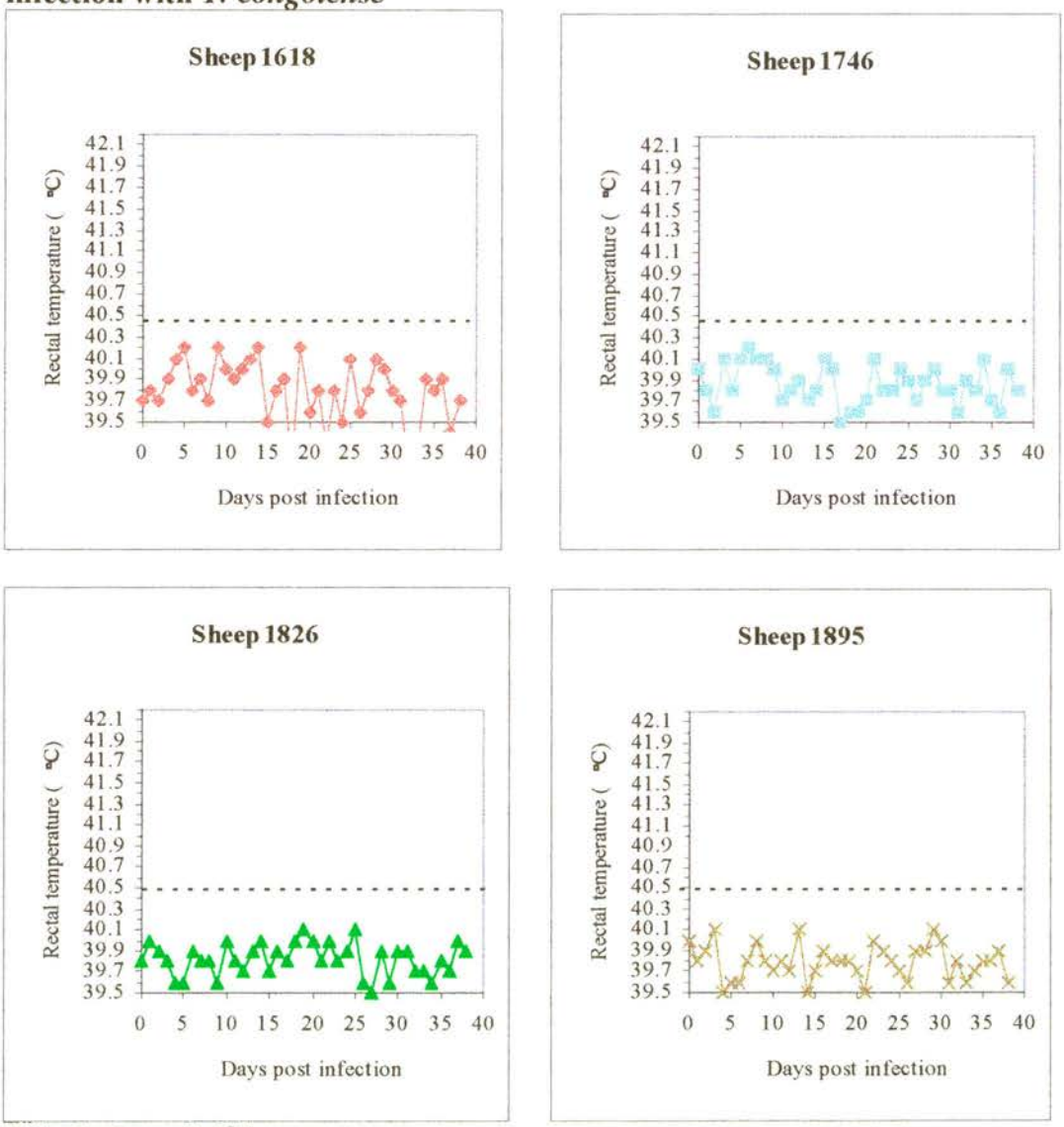
Figure 4.2 Rectal Temperature for the Control group following primary infection with *T. congolense*



Critical value = 40.5°C

None of the sheep in the prophylactic group developed fever and their rectal temperature values were all below the critical value of 40.5°C (Figure 4.3; Table A1(b)-Appendix II). The areas under the temperature time curve were; 300 mm^2 for sheep 1618, 225 mm^2 for sheep 1746, 75 mm^2 for sheep 1895 and 50 mm^2 for sheep 1826. The median value of 150 mm^2 was significantly different ($P<0.05$) from that of the control (4825 mm^2).

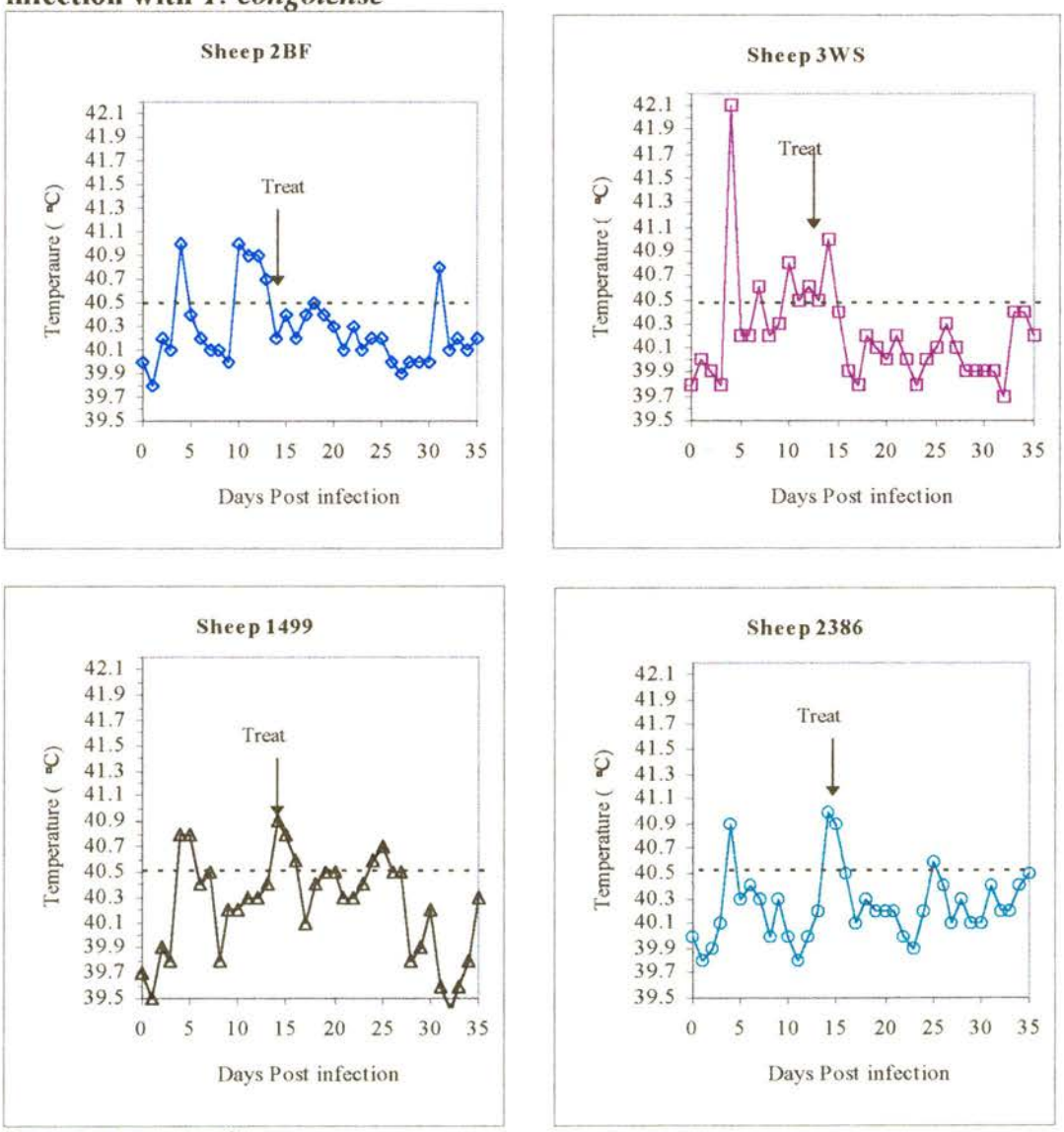
Figure 4.3 Rectal Temperature for the Prophylactic group following primary infection with *T. congolense*



Critical value = 40.5°C

The treated group also showed waves of fluctuating fever which subsided following treatment with ISMM (Figure 4.4; Table A1(c)-Appendix II). However, sheep 2BF became febrile again 31 days post infection. The magnitude was highest in sheep 1499 and lowest in sheep 3WS. The area under the curve being; 3025 mm^2 for sheep 1499, 2875 mm^2 for sheep 2BF, 2675 mm^2 for sheep 2386 and 2425 mm^2 for sheep 3WS. Median area under the curve from zero to 17 days post infection was 1688 mm^2 and dropped non-significantly ($P>0.05$) to 1350 mm^2 thereafter. The median area under the curve for the whole study period for the treated group of 2775 mm^2 was significantly ($P<0.05$) lower than that of the control group of 4825 mm^2.

Figure 4.4 Rectal Temperature for the Treated group following primary infection with *T. congolense*

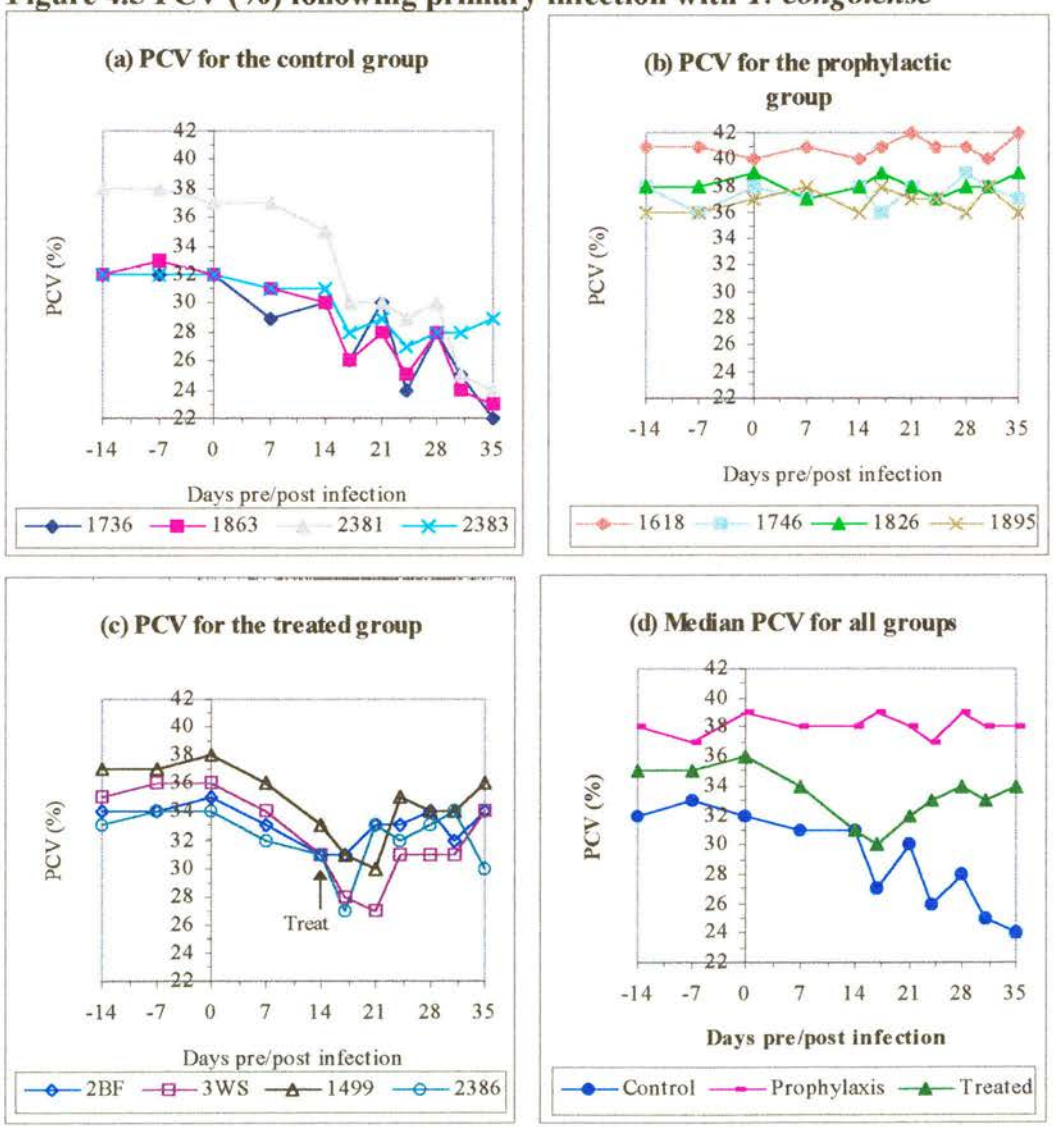


Critical value = 40.5°C

4.3.3 Packed cell volume (PCV, (%)) following primary infection with *T. congolense*

Figure 4.5 and Table A2-Appendix II, show results for the PCV. In the control group the PCV dropped significantly (P<0.0001) from a pre-infection median value of 32% to that of 22% thirty five days post infection, while no significant changes in the prophylactic group were observed. In the treated group, the PCV decreased significantly (P<0.0001) from a median of 35% to 28% 21 days post infection, but returned to normal about 3 weeks after treatment.

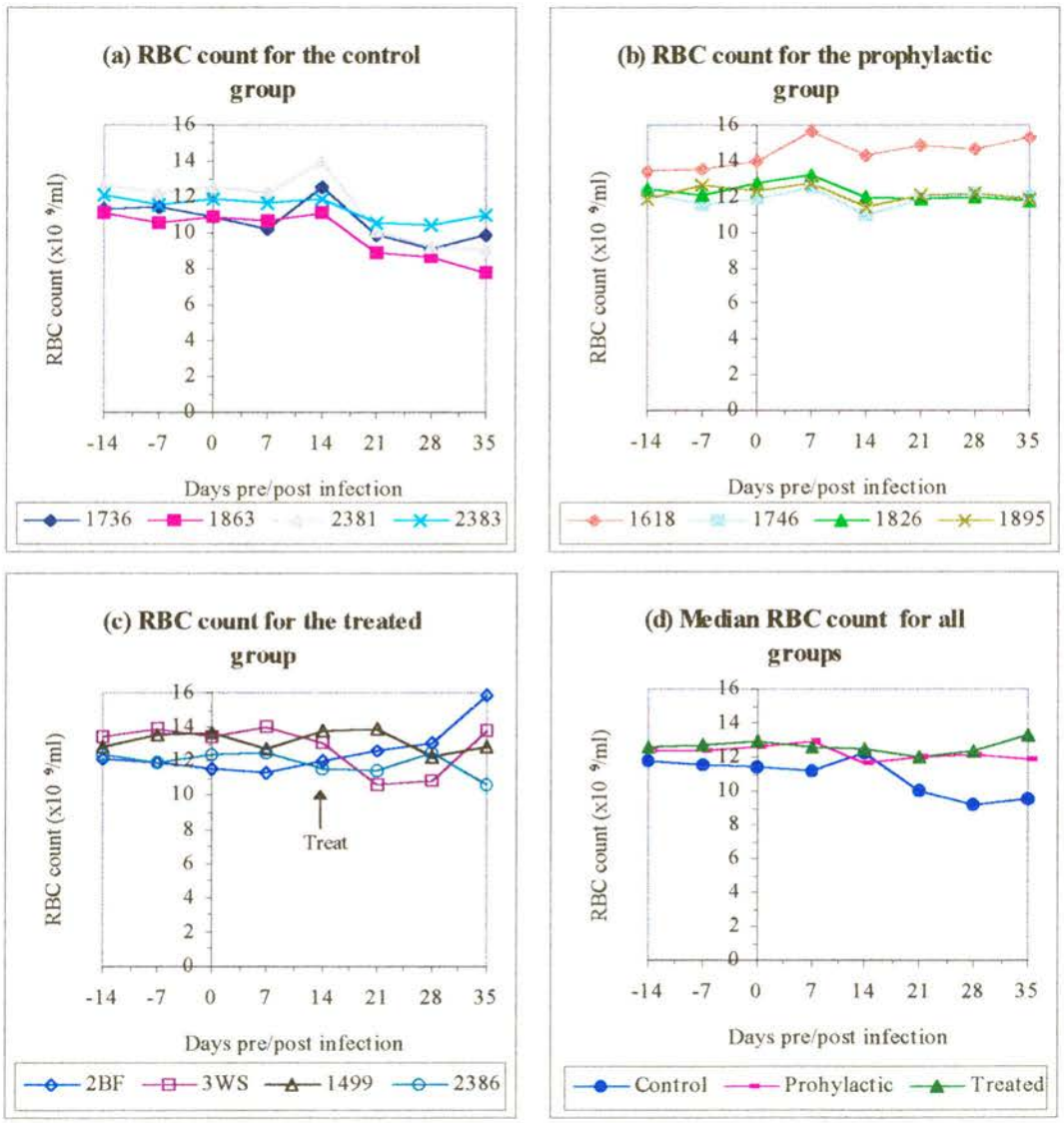
Figure 4.5 PCV (%) following primary infection with *T. congolense*



4.3.4 RBC count following primary infection with *T. congolense*

Results in Figure 4.6 and Table A3-Appendix II, show a significant loss of red cells in the control group 21 to 35 days post infection compared to pre-infection levels ($P<0.0001$). Despite, a decrease in the PCV in the treated group, RBC count remained unchanged throughout the period ($P>0.05$). No changes in RBC counts were recorded in the prophylactic group ($P>0.05$).

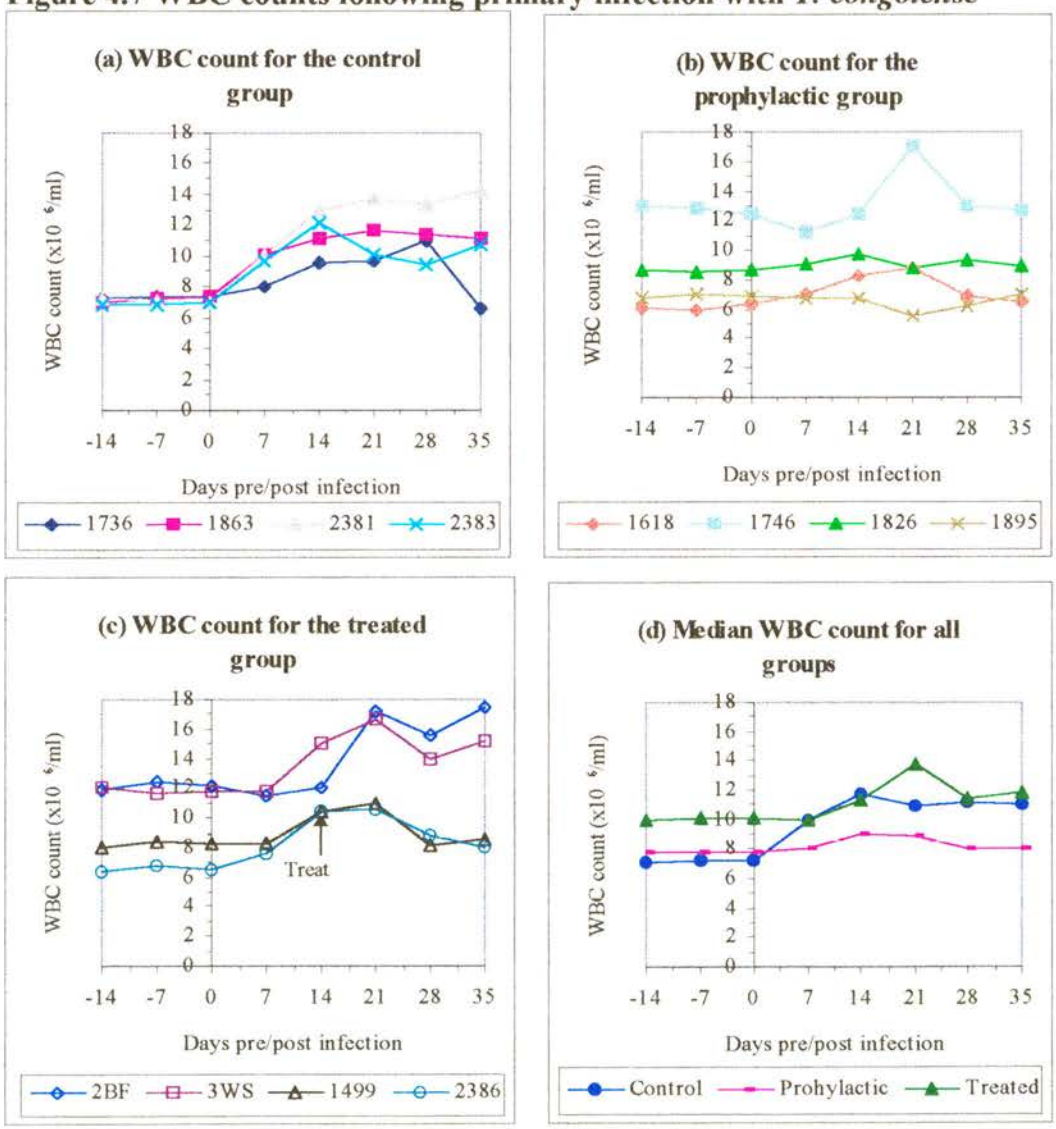
Figure 4.6 RBC count following primary infection with *T. congolense*



4.3.5 WBC counts following primary infection with *T. congolense*

WBC counts increased significantly ($P<0.0001$) in the control group from 14 to 35 days post infection compared to pre-infection levels (Figure 4.7; Table A4- Appendix II). Only sheep 1746 in the prophylactic group showed a non-significant transient increase 21 days post infection ($P>0.05$). No significant increase in the treated group was observed after infection ($P>0.05$). However, levels were slightly higher than normal for all sheep in the treated group, but returned to normal in sheep 1499 and 2386 after treatment.

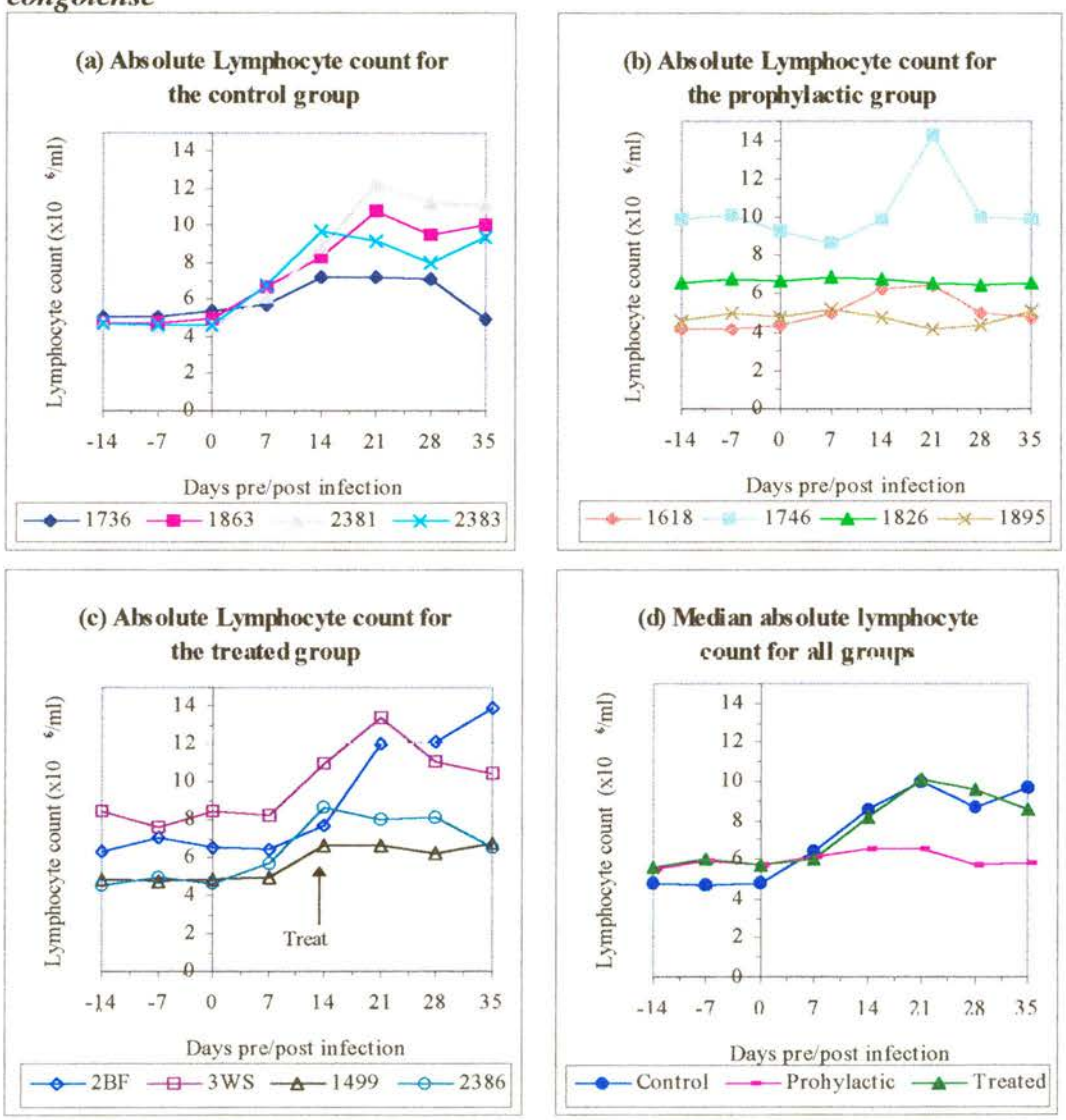
Figure 4.7 WBC counts following primary infection with *T. congolense*



4.3.6 Absolute Lymphocyte counts following primary infection with *T. congolense*

Absolute lymphocyte counts increased significantly ($P<0.0001$) in the control groups from 14 to 35 days post infection compared to pre-infection levels (Figure 4.8; Table A5-Appendix II). Only sheep 1746 in the prophylactic group showed a non-significant transient increase 21 days post infection. A significant increase was also observed in the treated group after infection ($P<0.01$).

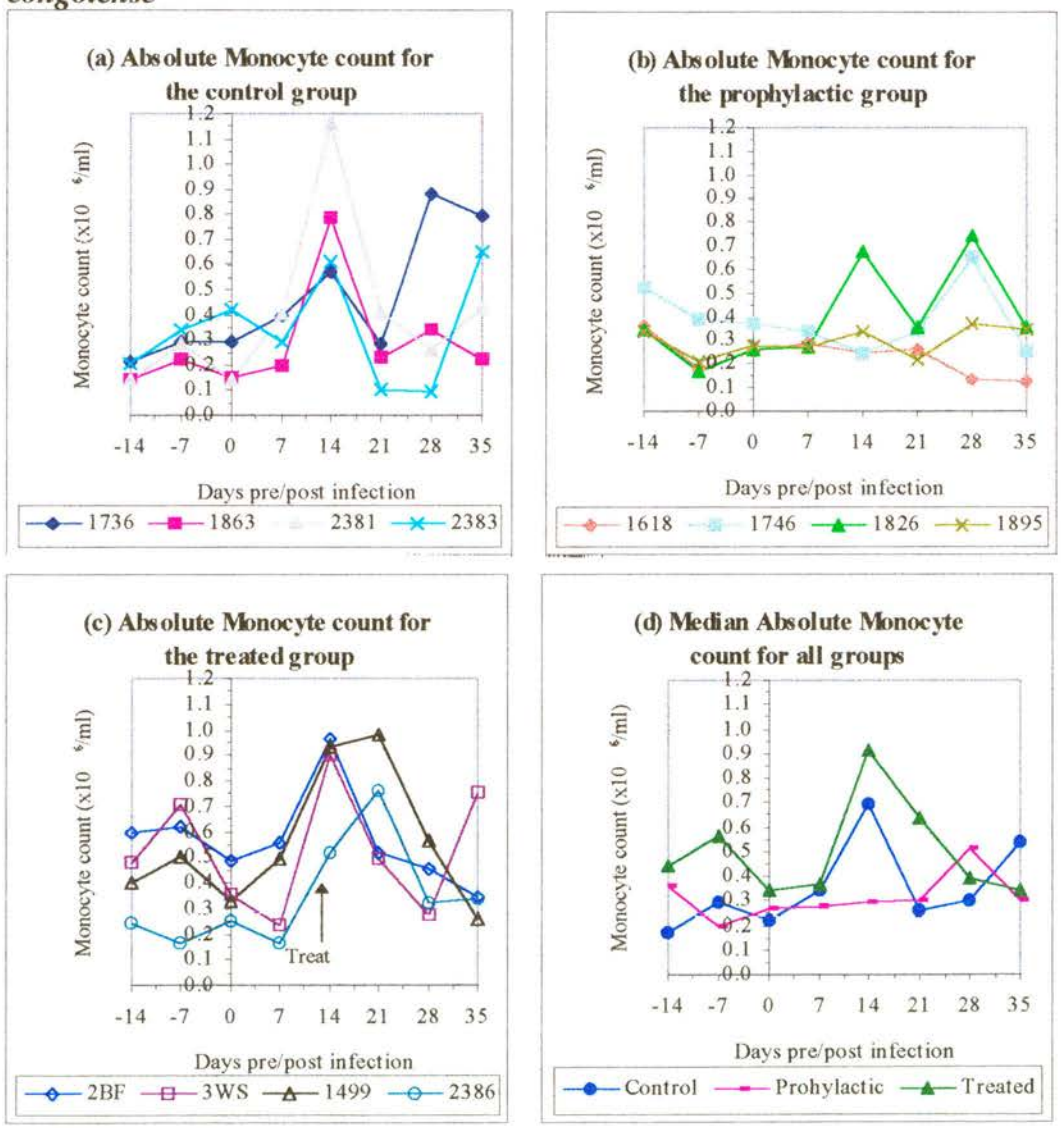
Figure 4.8 Absolute Lymphocyte counts following primary infection with *T. congolense*



4.3.7 Absolute Monocyte counts following primary infection with *T. congolense*

Figure 4.9 and Table A6-Appendix II, shows that the median monocyte count in the control group 14 to 35 days post infection was significantly different from that before infection ($P<0.05$). The highest increase was observed 14 days post infection. In the prophylactic group, the change was not significant ($P>0.05$), although sheep 1746 and 1826 seemed to show an increase 14 to 28 days post infection. A significant increase was recorded in the treated group 14 to 21 days post infection ($P<0.01$), but returned to normal about 2 weeks after treatment.

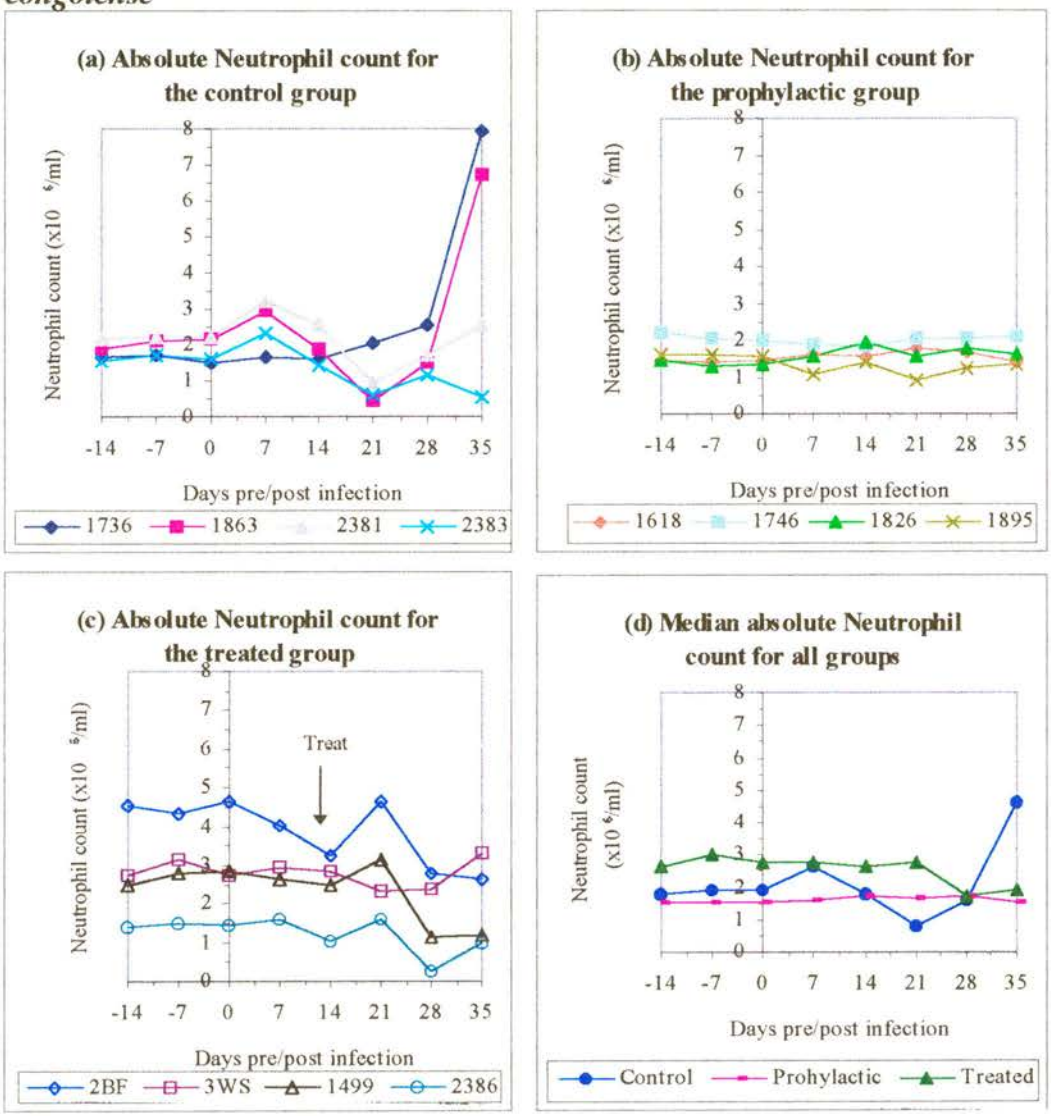
Figure 4.9 Absolute Monocyte counts following primary infection with *T. congolense*



4.3.8 Absolute Neutrophil counts following primary infection with *T. congolense*

Absolute neutrophil counts, did not show any significant changes (P>0.05) in all groups (Figures 4.10; Table A7-Appendix II), even in the control group where sheep 1736 and 1863 had high levels 35 days post infection.

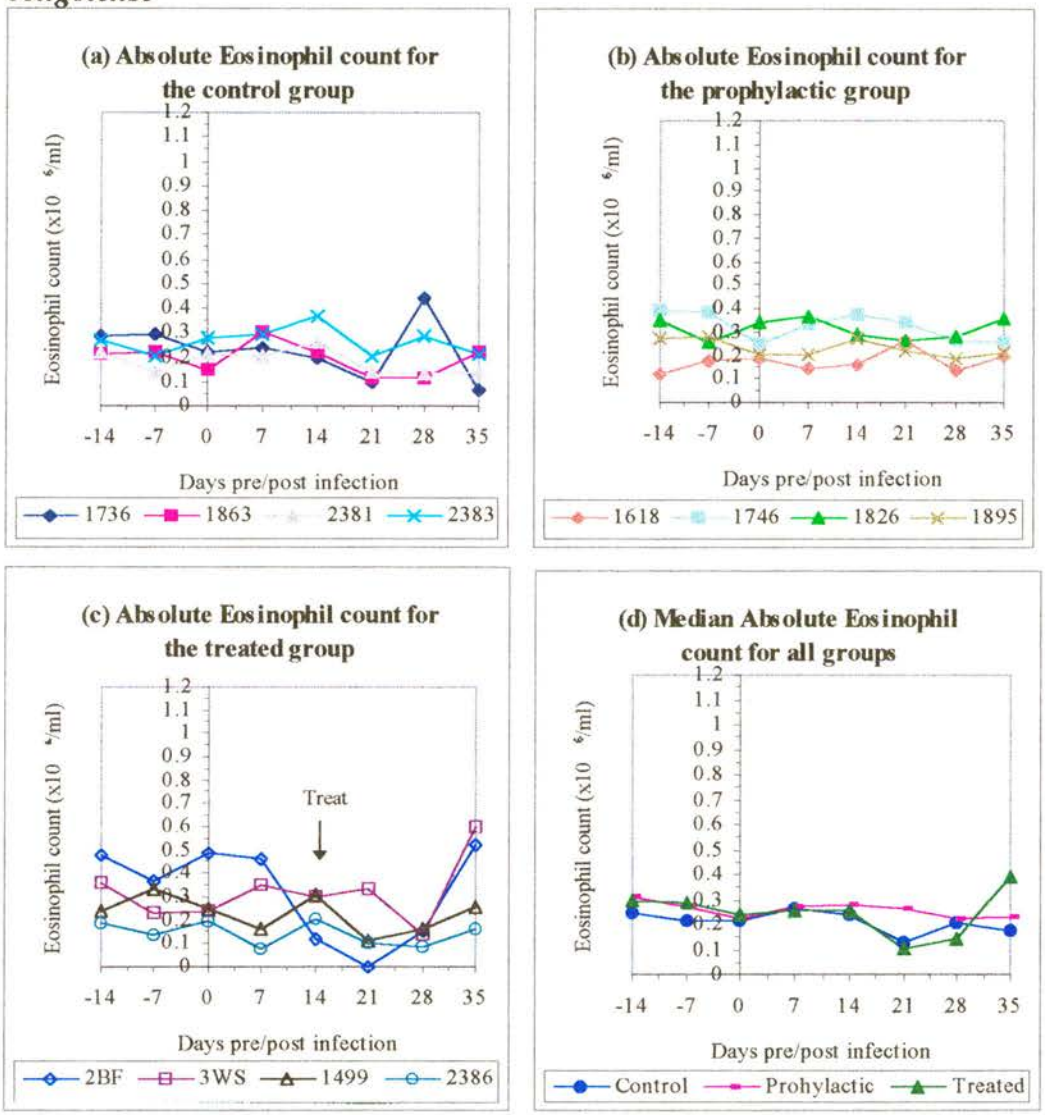
Figure 4.10 Absolute Neutrophil counts following primary infection with *T. congolense*



4.3.9 Absolute Eosinophil count following primary infection with *T. congolense*

Results summarised in Figure 4.11 and Table A8-Appendix II, indicate that absolute eosinophil counts, did not show any significant changes in all groups throughout the study period (P>0.05).

Figure 4.11 Absolute Eosinophil count following primary infection with *T. congolense*



4.4: DISCUSSION

Parasitaemia was determined using the haemotocrit method and results show that no sheep in the prophylactic group was parasitaemic throughout any of the study period, while all sheep from the control and treated groups became parasitaemic 7 days post infection. The control group remained parasitaemic, while after treatment parasites were cleared from the treated group, (although sheep 2BF relapsed 35 days post infection). Sheep 1863 and 2381 from the control group showed signs of jaundice (i.e. yellow mucous membranes and serum) 28 days post infection. This was an indication of severe destruction of red blood cells. Rectal temperature above the critical value of 40.5°C was used to determine fever and the area under the temperature time curve gave an estimate of the magnitude of fever. Results show that the magnitude of fluctuating fever were highest in the control group with a median area under the curve of 4825 mm^2 followed by the treated group with 2775 mm^2. The magnitude of fever in the treated group of 1688 mm^2, zero to 17 days post infection was not significantly higher than 1350 mm^2 that prevailed after treatment because although rectal temperature for sheep 1466 and 2386 was below the critical value it remained consistently above the baseline of 40°C used for calculating the area under the curve. Sheep from the prophylactic group were all not febrile and the median area under the curve was the lowest (150 mm^2). Fever usually fluctuates with waves of parasitaemia (Williams *et al* 1991; Onah 1990) and each peak of fever normally correspond to a peak of parasitaemia, but in this study it was not possible match fever with parasitaemia because parasitaemia was determined weekly while temperature was taken daily. Comparing N'dama and Boran cattle undergoing a primary infection with *T. congolense*, Williams *et al* (1991) found that parasitaemia was lower and usually intermittent in the trypanotolerant N'dama than in the susceptible Boran cattle. Results of this study demonstrate that a prophylactic dose of 1 mg/kg ISMM was capable of preventing the establishment of a *T. congolense* infection four half months after treatment and that a similar dose was also capable of clearing an established infection although relapses could occur in some cases. Complete protection was also afforded by 1 mg/kg ISMM for four months (Peregrine *et al* 1988), and for one to six months (Eisler *et al* 1994) in Boran cattle. Sones, Ngoju and Holmes (1988) assessed

the sensitivity of different strains of *T. congolense* to ISMM and found that certain strains of *T. congolense* could only be temporarily cleared from some animals.

Anaemia was assessed by measuring PCV and total red cell count. Both parameters decreased from 14 days post infection till end of experiment 35 days post infection in the control group. In the group that was treated with 1 mg/kg ISMM 14 days post infection, the PCV begun to return to normal around 25 days post infection. However, a change in total red cell count was not observed in the treated group even during the period of low PCV for yet unknown reasons. This result was unexpected, but probably the fluorescence properties of ISMM interfered with the electronic cell counting principle of the Coulter counter, after binding to RBCs, resulting in an overestimation of their count. For instance, an increase in background fluorescence was observed on a FASCAN when PBMCs had been incubated in more than 10 ng/ml ISMM. However, a blank for each sample on a FASCAN eliminates that effect, but it is not possible to do that on a Coulter counter for each sample. In the prophylactic group there was no change of the PCV as well as total red cell count. Anaemia is one of the major pathological lesions associated with animal trypanosomiasis. The principal factor in the causation of anaemia is accelerated red cell loss from the circulation (Jennings *et al* 1974; Mamo and Holmes 1975). The onset and degree of red cell loss are closely associated with the development and level of parasitaemia. The precise aetiology of anaemia is still obscure but four mechanisms are proposed. The currently favoured hypothesis is that antibodies specific for trypanosomes become complexed with antigen on the red cells leading their sequestration and destruction in the reticuloendothelial system (Kobayashi, Tizard and Woo 1976; Dodd *et al* 1978). The second possible cause of red cell loss my be haemolysis produced by trypanosomes, however, the specific nature of these haemolysins has not been characterised (Huan 1975; Tizard *et al* 1977). Other possible causes could be the consequence of a non-specific expansion of the mononuclear phagocytic system or an increased red cell fragility caused by fever. Trypanotolerant N'dama cattle have the ability to control the parasitaemia and hence the loss of red cells unlike most of the susceptible Zebu cattle. Dargie *et al* (1979) observed that the PCV decreased to a

steady state of 22% in Zebu and 27% in N'dama from a mean of 35%. However, parasitaemia in N'dama cattle fell to undetectable levels 5 weeks post infection while Zebu cattle entered a chronic phase of infection.

Total white blood cell (WBC) and absolute lymphocyte counts increased significantly in the control group 14 days post infection till end of experiment 35 days post infection. The pattern of increase in WBC counts is similar to that of absolute lymphocyte counts because lymphocytosis is the major contributing factor to increased WBC count. On the other hand, in the prophylactic group only two sheep (1746 and 1618) showed a transient rise 21 days post infection, resulting in a non-significant increase for the group. Levels of WBC and lymphocyte counts in sheep 2BF and 3WS from the treated group remained higher than normal throughout the period while those for sheep 1499 and 2386 returned to normal 2 weeks after treatment. Lymphocytosis and increased WBC counts were also demonstrated by Mwangi (1991) in sheep infected with *T. congolense* 25 days post infection onwards. In studies involving cattle infected with *T. congolense* (Williams *et al* 1991), lymphocytopoenia was observed in Boran cattle upto 14 days post infection, while N'dama cattle showed lymphocytosis from 28 days onwards. Lymphocytopaenia followed by lymphocytosis (Valli and Forsberg 1979) or continued lymphocytopoenia (Wellde *et al* 1974) have been documented in cattle infections with *T. congolense*.

Absolute monocyte counts increased in the control and treated groups 14 to 21 days post-infection and were almost normal thereafter in both groups. There were no significant monocyte changes in the prophylactic group. Absolute neutrophil and eosinophils counts, did not show any significant changes in all groups. It is possible that changes in neutrophil and eosinophil counts were missed because they are likely to occur before 7 days after infection, which happened to be the sampling interval in this study. Results of monocyte changes are not in agreement, while neutrophil and eosinophil counts are in agreement with those obtained by Mwangi (1991), where no changes in monocyte, neutrophil and eosinophil counts were observed in sheep. Williams *et al* (1991) observed a decline in neutrophil counts 7 to 14 days after

infection in Boran but not in N'dama cattle, but were unable to find significant changes in eosinophil counts in both breeds over time but found some increases in monocytes without any breed differences.

In conclusion, these results demonstrate that ISMM prophylaxis inhibited the establishment of a *T. congolense* infection four and a half months after treatment, thereby preventing the development of major pathological lesions associated with the disease such as anaemia, lymphopaenia and polyclonal lymphocytosis.

CHAPTER 5

CHAPTER 5
IN VITRO AND *IN VIVO* EFFICACY OF ISOMETAMIDIUM PROPHYLAXIS

5.1 INTRODUCTION

It is generally understood that therapeutic agents against trypanosomiasis either kill the parasites (trypanocidal) or simply suppress their multiplication (trypanostatic). Some agents can be trypanocidal at high concentrations and trypanostatic at low concentrations. Therefore, without an effective immune response trypanostatic agents are quite ineffective. ISMM is considered to be trypanostatic at the prophylactic dose. Despite this understanding, several studies seem to state that ISMM is trypanocidal and rule out the involvement of the immune system (Whitelaw *et al* 1986; Peregrine *et al* 1988) simply because they were not able to detect neutralising antibodies. This is quite misleading and needs to be seriously redressed.

Parameters which influence the effectiveness of ISMM have not been well defined, as a result a considerable uncertainty remains as to the most effective way to use the drug in the field. The study of combined effects of chemotherapeutic agents and the immune system against infection remain in its infancy. Experimental evidence exists which shows that the immune system plays a significant role in the effectiveness of difluorometlylornithine (DFMO) chemotherapy in African trypanosomiasis in mice (DeGee, MacCann and Mansfield 1983). Also, repeated sub-curative drug treatment with cymerlarsan, berenil or ISMM was found to lead to the development of drug resistant trypanosomes only in immunosuppressed animals (Osman, Jennings and Holmes 1992). These results suggest that the state of the host immune system may play an important role in the development of drug resistance. However, the role played by the immune system on the efficacy of isometamidium, the sole prophylactic drug against livestock trypanosomiasis is not well understood. The relationship between immunocompetence and prophylaxis is of great clinical importance since it is known that trypanosome infection and other stress factors are associated with immunosuppression.

Breakdowns in isometamidium prophylaxis have been frequently observed in the field but it is not usually clear whether these are due to inadequate dosing, poor immune status of the host or to the development of drug resistant trypanosomes. The duration of prophylaxis varies from as little as 2 weeks (Dolan *et al* 1992, Munstermann *et al* 1992) to 14 months (Wellde *et al* 1973). The relative contribution of drug resistance, variability in pharmacokinetics and the state of the host's immune system to the variation in the duration of prophylaxis could not be determined previously due to the lack of a method capable of measuring low ISMM plasma or serum levels during the prophylactic period. An enzyme-linked immunosorbant assay (ELISA) has been developed (Whitelaw *et al* 1991) for the measurement of ISMM levels in sera of treated cattle and has made assessment of contributing factors possible.

The objective of this experiment was to: (a) establish ISMM plasma levels prevalent during the protective period. (b) determine trypanocidal effects of ISMM concentration present during the protective period. (c) establish the role played by the innate immunity during ISMM prophylaxis.

5.2 MATERIALS AND METHODS

Methods for determining the profile of ISMM concentration in plasma, *in vitro* and *in vivo* efficacy of ISMM are described in Chapter 3, sections 3.4 and 3.5.

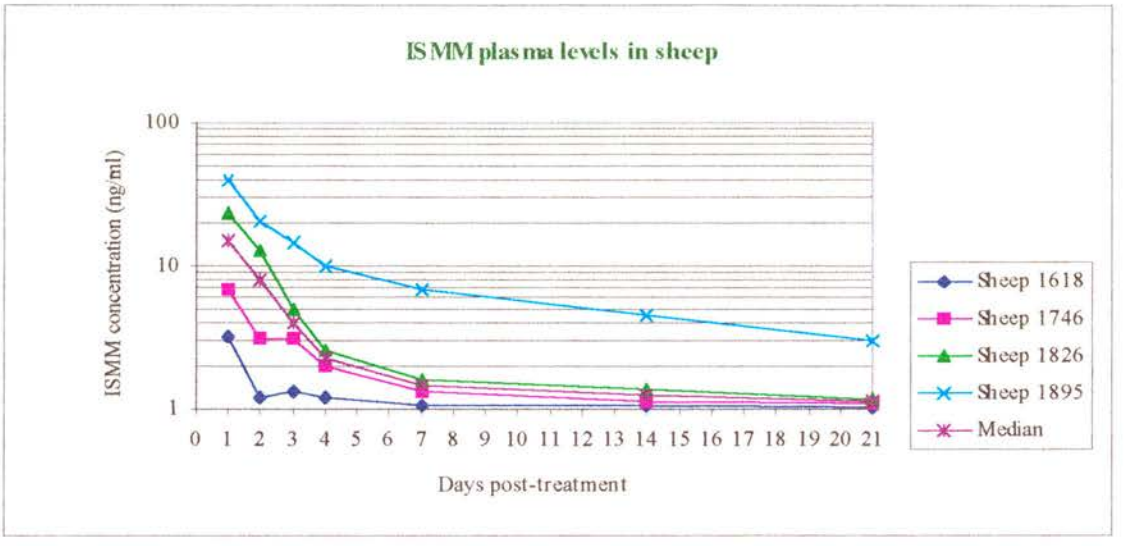
5.3 RESULTS

5.3.1 ISMM plasma profile

The concentration of ISMM was extrapolated from the standard absorbance plotted against the logarithm of the concentration. A semi-logarithmic plot of concentration against time yielded a biexponential graph showing that the bioavailability of ISMM varied greatly between animals although the elimination process was very similar in all animals. The elimination of the drug was bi-phasic in all animals with a rapid elimination rate in the first week followed by a slow phase there after. The mean

ISMM levels were 2.7 ng/ml (range 1.1-6.9 ng/ml) after one week (Figure 5.1; Table B1-Appendix III).

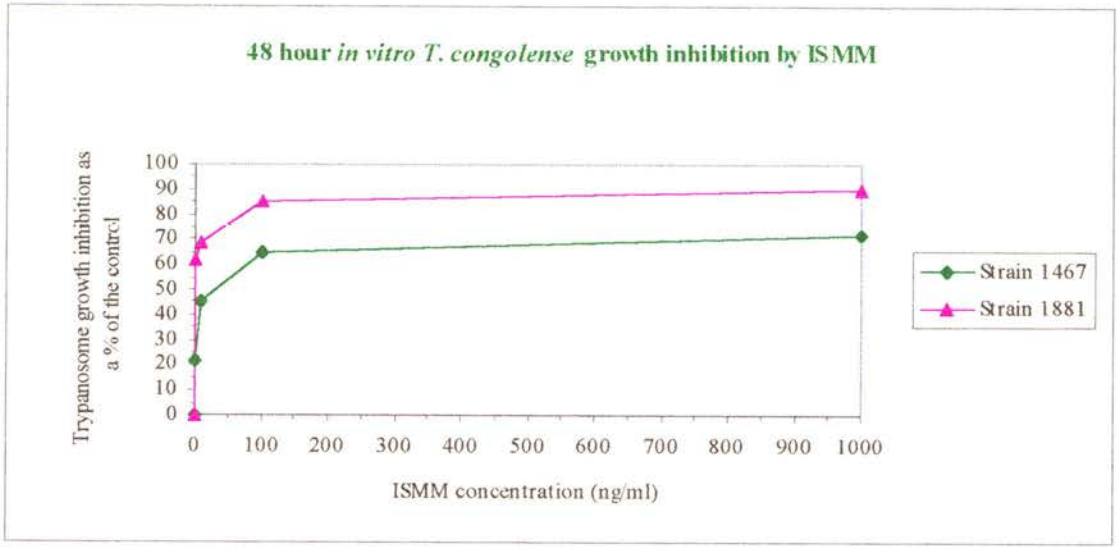
Figure 5.1 ISMM plasma profile



5.3.2 *In vitro* sensitivity of *T. congolense* to ISMM

Blood stream forms of *T. congolense* isolates TREU1467 and TREU1881 were cultured *in vitro* at an initial concentration of 5 x 10^5 trypanosomes/ml for 48 hours in increasing concentrations of ISMM. After a 48 hour incubation period, viable trypanosomes were estimated using the Promega® cell proliferation assay. Inhibition of trypanosome growth was calculated as a percentage of the control cultures (media only) subtracted from 100 since assay readings corresponded to the number of viable, and not dead cells present. Results show that the percentage inhibition of *T. congolense* multiplication nearly reached a maximum of 72% for isolate TREU1467 and 85% for TREU1881 at the same concentration of approximately 100ng/ml. Thereafter any increase in drug concentration produced very little inhibition. At concentrations less than 10 ng/ml the inhibitory effect was less than 45% for isolate TREU1467 and less than 69% for TREU1881 (Figure 5.2; Table B2-Appendix III).

Figure 5.2 *In vitro* sensitivity of *T. congolense* to ISMM



5.3.3 Prepatent period and survival time in mice infected with *T. congolense*

Four groups consisting of 20 mice were used in this experiment. Group one was a control, group two received 0.02 mg hydrocortisone daily for four consecutive days, group three received a single prophylactic dose of 1 mg/kg ISMM. Group four was given a single prophylactic dose of 1 mg/kg ISMM and 0.02 mg hydrocortisone daily for four consecutive days. Eleven days post ISMM treatment all groups were inoculated with 10^6 trypanosomes intraperitoneally. Thereafter they were examined for parasitaemia, pre-patent period and survival time. Results show that the prepatent period (Figure 5.3; Table B3-Appendix III) and the survival time (Figure 5.4; Table B4-Appendix III) for the control, hydrocortisone and ISMM plus hydrocortisone groups were not significantly different ($P>0.05$). However, the prepatent period for the ISMM only group was significantly longer than that of the ISMM plus hydrocortisone group ($P<0.01$). The survival time in the ISMM group varied widely but was in general longer than that observed in the other groups.

Figure 5.3 Median prepatent period in mice infected with *T. congolense*

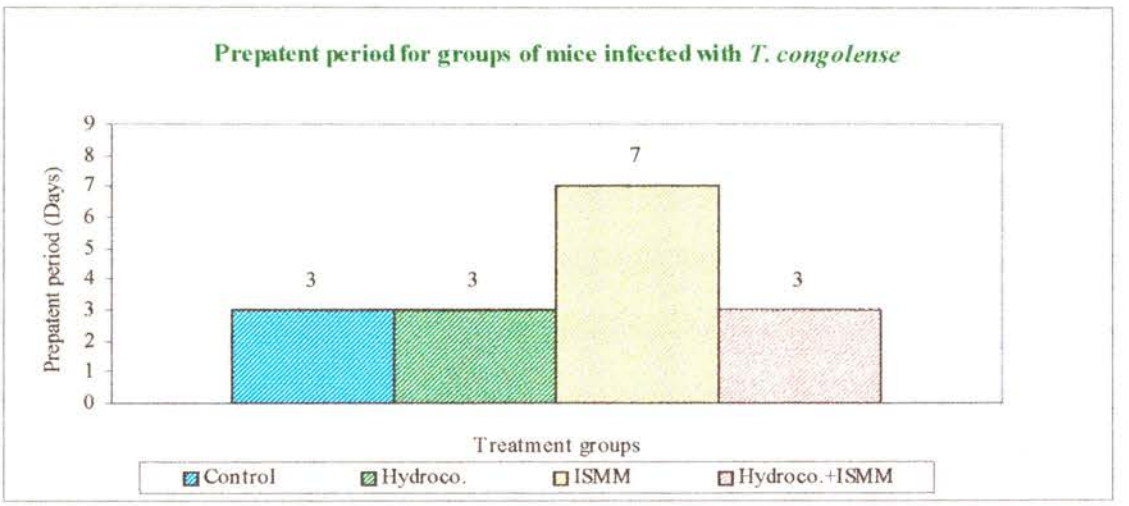
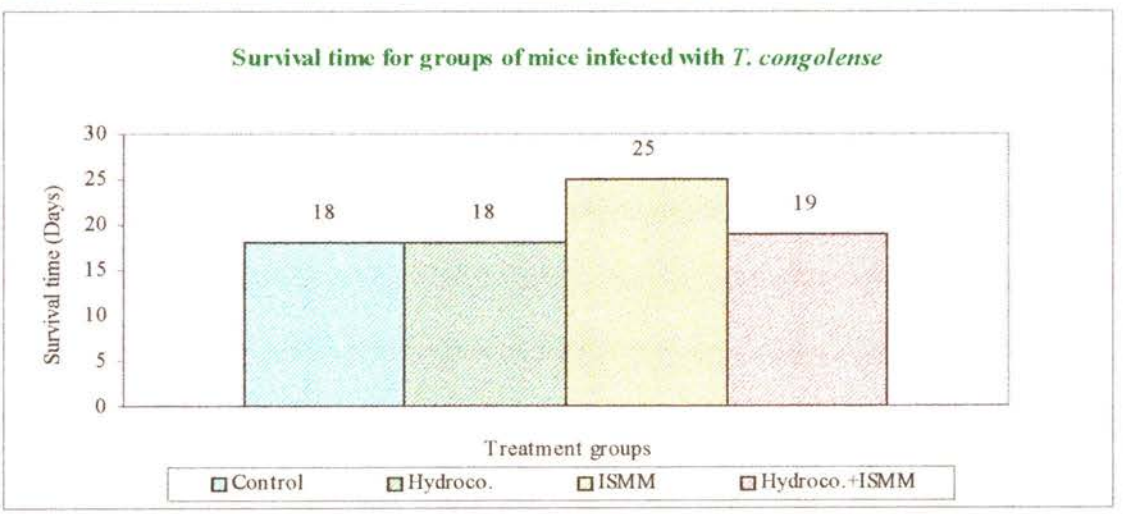


Figure 5.4 Median survival time for mice infected with *T. congolense*



ISMM = Isometamidium Hydroco. = Hydrocortisone

5.4 DISCUSSION

After administering 1 mg/ml of ISMM to four sheep, a semi-logarithmic plot of concentration against time yielded a biexponential graph. The bioavailability of ISMM varied greatly between animals and the drug concentration 24 hours after administration ranged from 3.21 to 40.52 ng/ml, with a median value of 18.48 ng/ml. Elimination of the drug was bi-phasic in all animals with a rapid elimination rate in the first week followed by a slow phase there after. By the end of the first week the median plasma ISMM levels were 2.7 ng/ml (range 1.1-6.9 ng/ml). Three weeks post treatment drug levels had dropped very significantly to a median of 1.5 (range 1 - 3 ng/ml). Despite these low drug levels all sheep were protected when challenged with *T. congolense* four and half months later. A similar kinetic profile was observed in cattle following ISMM administration (Eisler *et al* 1994). In that experiment the highest ISMM concentrations were observed at the time of first sampling 24 hour after administration were 40 -50 ng/ml. Serum concentrations fell rapidly for the first 8 days and rather more gradually thereafter. At the end of one month the mean concentrations was 6.1 ng/ml (range 2.8 - 12 ng/ml). All animals were protected when challenged with *T. congolense* one month later. In an experiment by Geerts *et al* (1997), serum mean ISMM levels were less than 10 ng/ml 24 hours after administration and they had dropped to undetectable levels after 90 days. However, all animals were protected from *T. congolense* infection for an average period of 5.7 months. In all of these experiments ISMM levels in plasma or serum were assayed using the ISMM-ELISA assay (Eisler *et al* 1994). These results demonstrates that even though the drug concentration can be very low or undetectable, ISMM provides protection against *T. congolense* infection in cattle as well as in sheep.

In order to determine the trypanocidal effects of ISMM concentrations prevalent during the protective period *in vitro*, two strains of *T. congolense* (TRUE 1467 and TRUE 1881) were cultured for 48 hours in increasing concentrations of ISMM. Results showed that the percentage inhibition of *T. congolense* multiplication plateaued at 72% for isolate TREU1467 and 85% for TREU1881 at the same

concentration of approximately 100 ng/ml. Thereafter, any increase in drug concentration produced very little further inhibition. At concentrations less than 10ng/ml the inhibitory effect was less than 45% for isolate TREU1467 and less than 69% for TREU1881. In addition, at the end of the 48 hour incubation period some viable trypanosomes were still present in all cultures including those at 1000 ng/ml. Related experiments have shown that culturing of different strains of *T. congolense*, *in vitro* in the presence of 5 ng/ml ISMM for about 10 minutes (Sutherland, Mounsey and Holmes 1991), 10 ng/ml for about 24 hours (Kaminsky, Chuma and Wasike 1994) and 10 ng/ml for about 48 hours (Gray and Peregrine 1993) could not completely eliminate the parasites or prevent infection. In this study strain TREU 1881 was used to infect the control and ISMM treated sheep with plasma profiles discussed above. These results demonstrate that the concentration of ISMM that was protective against *T. congolense* strain TREU 1881 *in vivo*, was incapable of eliminating the same strain *in vitro* after 48 hours of incubation. Since drug residues alone seem not be sufficient to protect animals against *T. congolense* infection, then the immune system is the most likely factor. Protection by ISMM is non-specific and does not depend on previous knowledge of the invading variant of trypanosome. This implies that the innate immunity is most likely to be factor potentiating actions of ISMM.

An experiment was set up to investigate whether some aspects of the innate immunity potentiates actions of ISMM *in vivo*. One group of mice was used as a control, the second group received 0.02 mg hydrocortisone daily for four consecutive days, the third one received a single prophylactic dose of 1 mg/kg ISMM. Group four was given a single prophylactic dose of 1 mg/kg ISMM and 0.02 mg hydrocortisone daily for four consecutive days. Eleven days post ISMM treatment all groups were inoculated with 10^6 trypanosomes intraperitoneally. Results show that the prepatent period and the survival time for the control, hydrocortisone and ISMM plus hydrocortisone groups were not significantly different. However, the prepatent period for the ISMM only group was significantly longer than that of the other groups. The survival time in the ISMM group varied widely but was in general longer than that

observed in the other groups. Results showed a median prepatent period of 3 days for the control and 7 days for the ISMM group, while that for ISMM plus hydrocortisone was 3 days. This implies that suppressing T cell responses by hydrocortisone reduced the ability of ISMM to control the establishment of the infection. In addition even though all mice developed parasitaemia and died, those in the ISMM only group had lower parasitaemia and survived longer than all the other groups including the ISMM plus hydrocortisone. However, the prepatent period for the ISMM only group was significantly longer than that of the other groups. The survival time in the ISMM group varied widely but was in general longer than that observed in the other groups. It is known that the dose of ISMM required to prevent or treat *T. congolense* infection effectively in mice is about ten times that for ruminants (Sones, Njogu and Holmes 1991). In this experiment the same dose on a body weight basis (1 mg/kg) was used in order to make comparisons relevant.

Hydrocortisone is a natural glucocorticoid hormone. Higher than normal levels can be produced during *T. congolense* infection (Mutayoba, Eckersall and Cestnik 1995 and Ogwu, Njogu and Ogbogu 1992) and under stress conditions leading to anti-inflammatory and immunosuppressive effects. The major immunosuppressive effects include; a decrease in concentration of various complement components, reduced proliferative responses to mitogens and antigens *in vitro*, decreased cytokine production (e.g. IL-2, IL-1) and decreased sensitivity of macrophages to cytokines resulting in decreased phagocytosis. Mice that received both ISMM and hydrocortisone exhibited no form of resistance to infection compared to those that were given ISMM alone which not only had a longer prepatent period but also had lower parasitaemia and lived longer.

It is therefore, concluded that ISMM drug concentration present during the prophylaxis period is not trypanocidal *in vitro* and the effectiveness of ISMM prophylaxis *in vivo* depends on the responsiveness of the innate immunity which potentiates its actions.

CHAPTER 6

CHAPTER 6
CELLULAR AND ANTIBODY RESPONSES TO PRIMARY *T. CONGOLENSE* INFECTION

6.1 INTRODUCTION

In Chapter 5 it was shown that ISMM prophylaxis was not effective at preventing *T. congolense* infection when the innate immune system was suppressed with hydrocortisone. Until it became possible to study detailed T cell responses in domestic animals, immunity to trypanosome infection was largely considered to be antibody dependent. For this reason very little had been done to investigate cell-mediated immune responses to trypanosomiasis. However, the little evidence that was available till the late 1970's suggested that cell-mediated immune responses were either absent or did not play a significant protective role (Campbell, Esser and Phillips 1978; Clayton, Ogilvie and Askonas 1979; Askonas *et al* 1979; Takayanagi and Nakatake 1975). This was largely based on knowledge obtained from studies in rodents, especially mice.

Studies in ruminants have provided evidence for T-cell-mediated responses to VSG and to invariant trypanosome antigens in cattle. Decreases in $CD4^+$ and $CD8^+$ T-cell subsets have been observed in cattle due to *T. congolense* infection (Lutje *et al* 1995) and in sheep due to *T. evansi* infection (Onah 1992). Both Th_1 and Th_2 responses have now been demonstrated in mice. The type of response seems to depend on the manner in which the antigen is presented. When purified VSG are administered a mixed response was obtained but if VSG was administered by way of live trypanosomes, only Th_1 response were observed (Mansfield 1994). Although, effector T cells may have no direct role in destroying trypanosomes, significant changes in their regulatory function might have a profound effect on the function of the whole immune system. It is for that reason that this chapter discusses lymphocyte phenotypic responses in the control and treated sheep as well as those prophylactically treated with ISMM. The final goal was to identify any responses that were characteristic of prophylactically treated animals and hence associated with resistance to infection.

6.2 MATERIALS AND METHODS

PBMCs were prepared and $CD4^+$, $CD5^+$, $CD8^+$ $\gamma\delta$ and B cell were analysed and trypanosome specific IgG antibody titre assayed as described in Chapter 3; sections 3.7, 3.8. and 3.12.

6.3 RESULTS

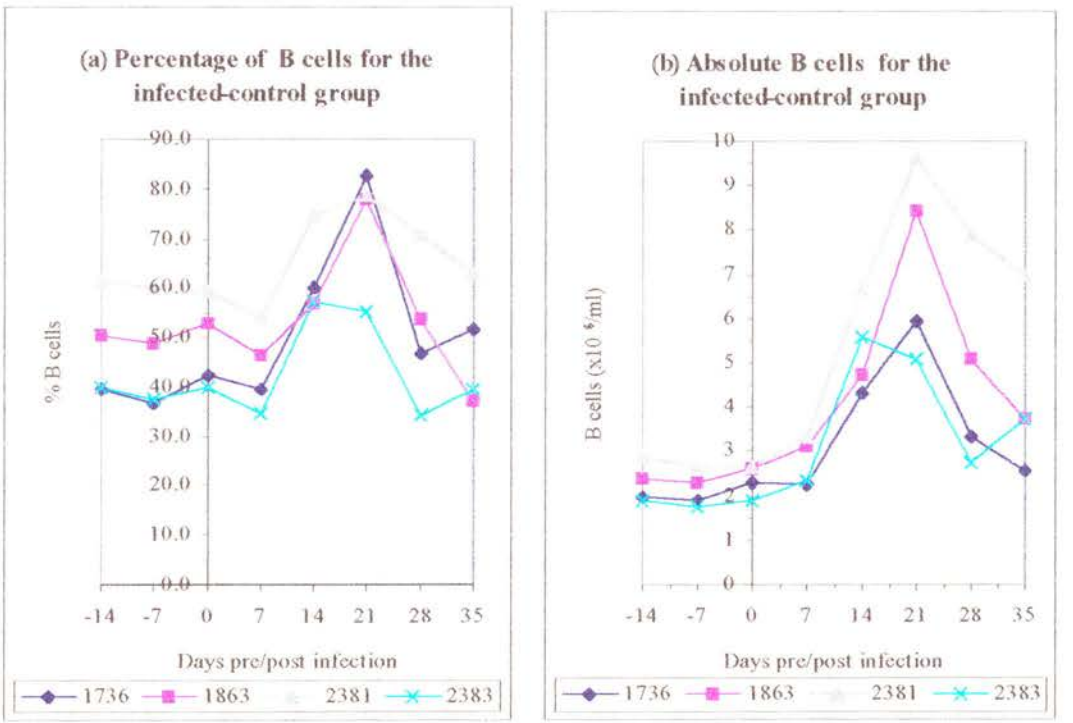
6.3.1 Flow cytometry histogram results

In the prophylactic group and control group, cellular phenotypes were analysed weekly after drug administration even before the infection in order to investigate any changes associated with the treatment. However, there were no observed cellular changes during the pre-infection period. Significant changes were obtained only after infection and representative flow cytometry histogram results for each phenotype and group are shown in Figures C1-C15; Appendix IV. Phenotypes obtained as percentages of mononuclear cells were multiplied by the absolute numbers of lymphocytes to get the absolute number for the particular phenotype.

6.3.2 B cell responses

All animals in the control group showed a significant increase in the percentage and absolute number of B cells 14 - 28 days after *T. congolense* infection peaking on day 21 (Figure 6.1; Table C1.1 and 1.2 -Appendix IV). The increase in the absolute number of B cells from a median of 2.2 x 10^6/ml of blood 7 days before infection to 7.3 x 10^6/ml 21 days post infection was statistically significant ($P<0.05$). Sheep 1863 and 2381 showed the highest increases in cell numbers followed by sheep 1736 and 2383.

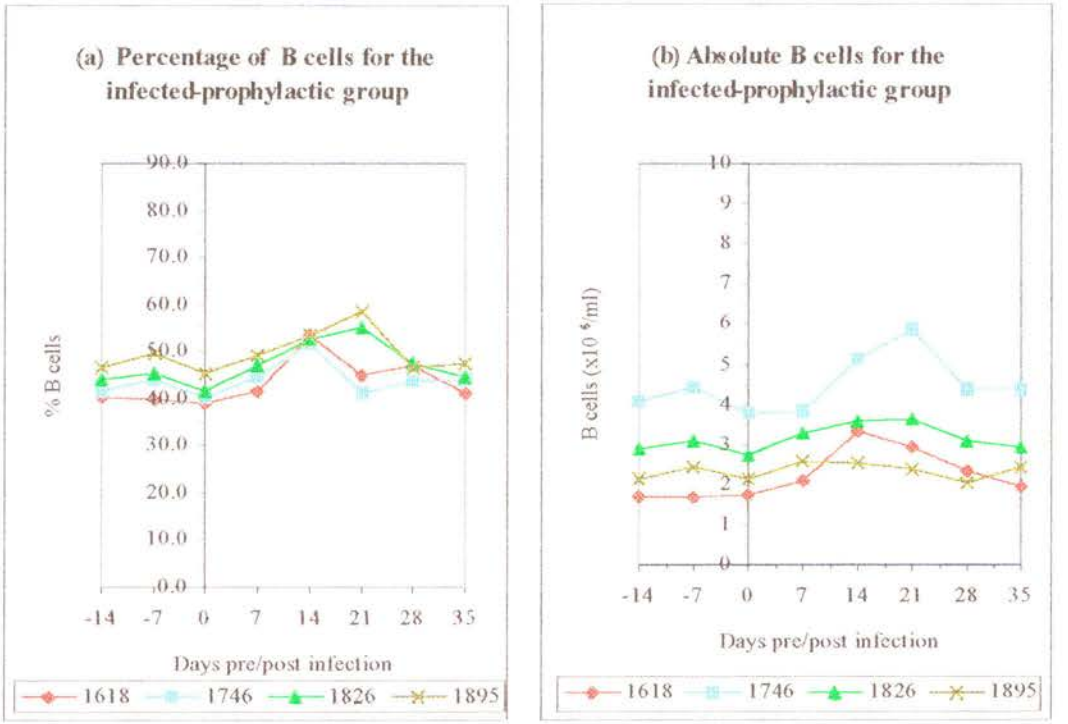
Figure 6.1 B cell responses in the control group



- All sheep were infected with *T. congolense* on day zero

The percentage and absolute number of B cells in the prophylactic group did not show any significant increase following *T. congolense* infection (Figure 6.2; Table C1.1 and 1.2 -Appendix IV). However, sheep number 1746 and 1618 showed a small increase 14 - 21 days after infection, while no detectable changes were observed for sheep 1826 and 1895.

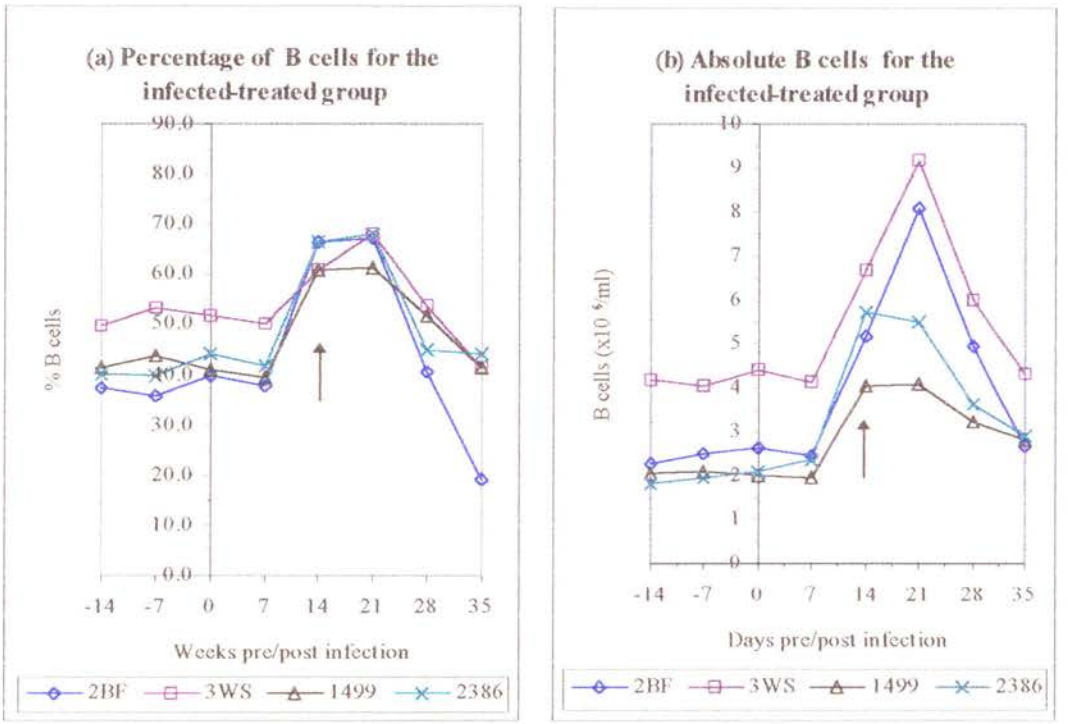
Figure 6.2 B cell responses in the prophylactic group



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered $4^{1}/_{2}$ months before infection

Absolute B cell counts in the treated group increased significantly from 14 days after infection onwards (Figure 6.3; Table C 1.1 and 1.2 - Appendix IV). The increase was from a median of 2.7 x 10^6 /ml of blood 7 days before infection to 6.7x10^6 /ml ($P<0.05$) 21 days afterwards. Treatment with ISMM 14 days after infection prevented any further increase in sheep 1499 and 2386 while in sheep 2BF and 3WS B cell numbers continued to increase in the same way as in the control group. However, by day 35 B cell numbers had returned to normal levels.

Figure 6.3 B cell responses in the treated group

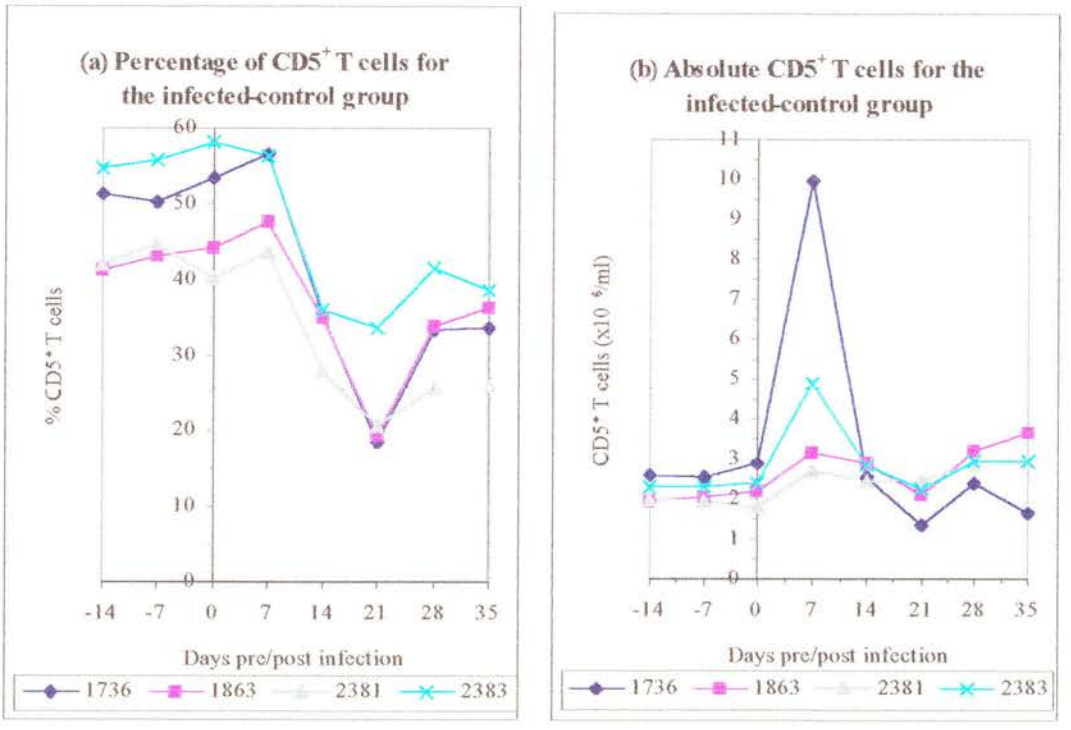


- All sheep were infected with *T. congolense* on day zero
- All sheep were treated with ISMM 14 days after infection (arrow)

6.3.3 $CD5^+$ T cell responses

In the control group the percentage of $CD5^+$ T cell started decreasing 14 days onwards while absolute numbers increased transiently 7 days after infection. An increase in absolute numbers of $CD5^+$ T cells was from 2 x 10^6/ml, 7 days pre-infection to 5 x 10^6/ml blood 7 days post infection. However, that increase was not statistically significant ($P>0.05$). The increase was highest for two sheep 1736 and 2383 and marginal for sheep 1863 and 2381, hence the nonsignificant of the results (Figure 6.4; Table C2.1 and 2.2 - Appendix IV).

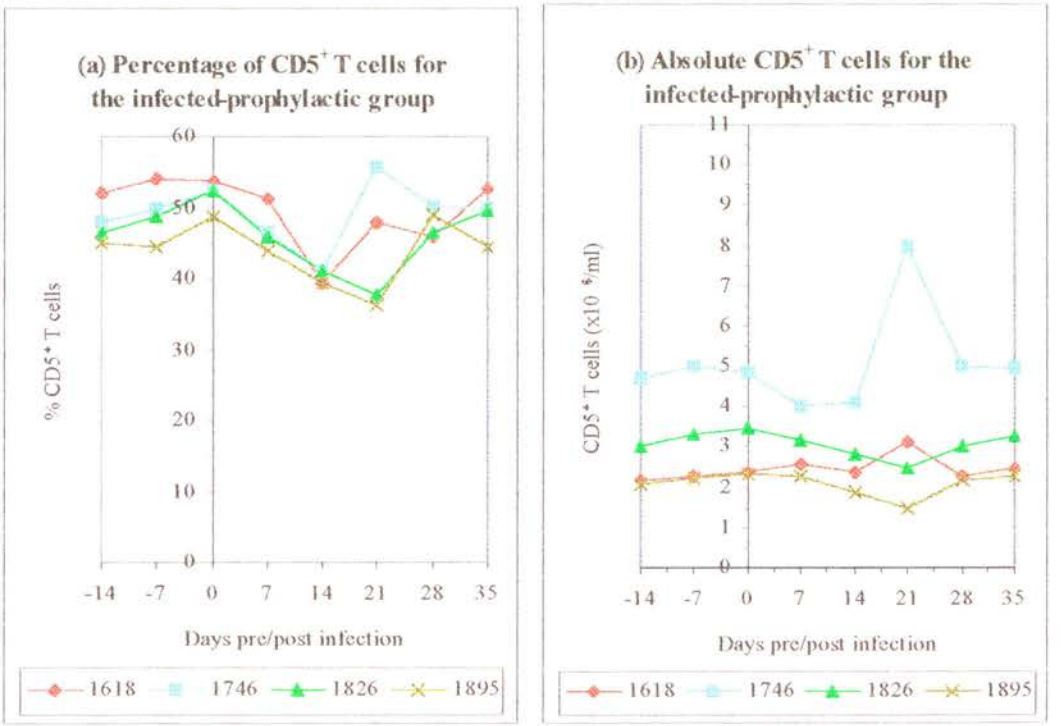
Figure 6.4 $CD5^+$ T cell responses in the control group



- All sheep were infected with *T. congolense* on day zero

All sheep in the prophylactic group showed no changes in absolute $CD5^+$ T cells after infection, except sheep 1746 which showed an increase 21 days post infection (Figure 6.5; Table C2.1 and 2.2 - Appendix IV).

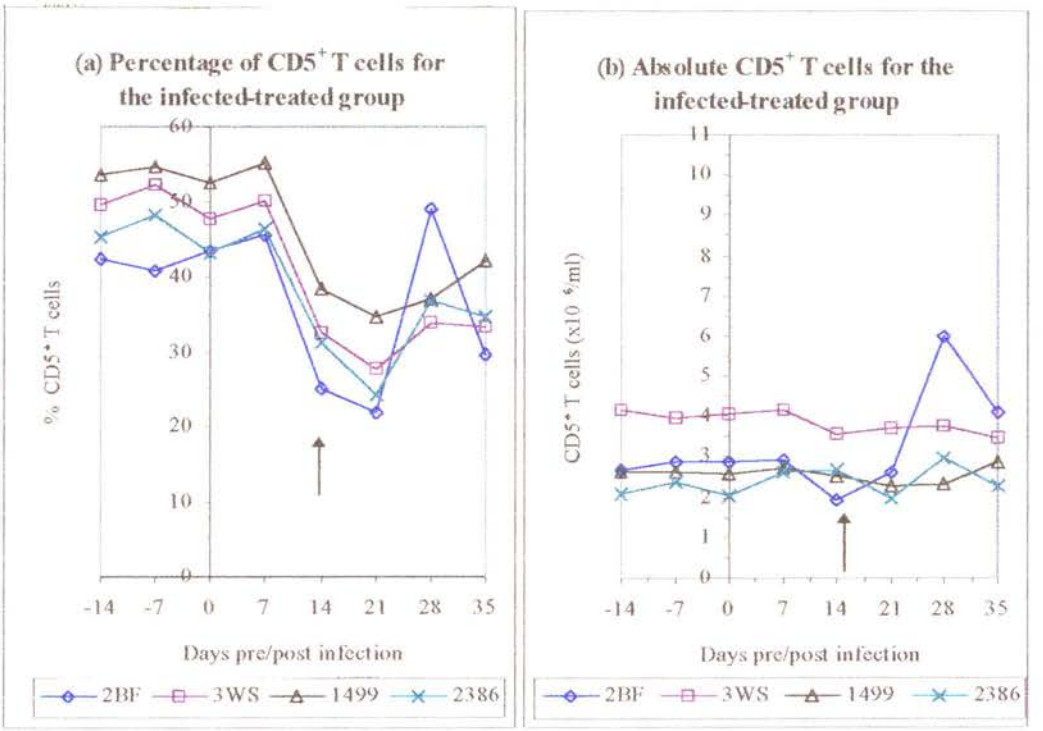
Figure 6.5 $CD5^+$ T cell responses in the prophylactic group



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered $4^1/_2$ months before infection

None of the sheep in the treated group showed any significant changes in absolute $CD5^+$ T cells through out the experimental period although sheep 2BF exhibited a slight transient increase 28 days after infection (Figure 6.6; Table C2.1 and 2.2 - Appendix IV). However, the proportions decreased from 14 days onwards.

Figure 6.6 $CD5^+$ T cell responses in the treated group

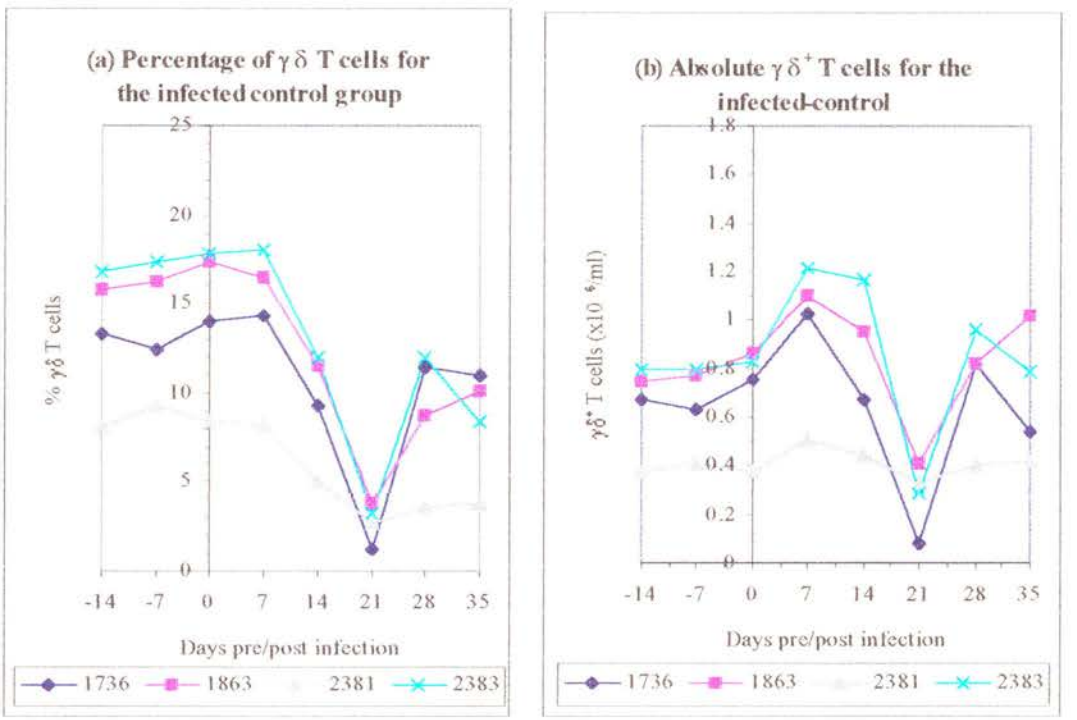


- All sheep were infected with *T. congolense* on day zero
- All sheep were treated with ISMM 14 days after infection (arrow)

6.3.4 $\gamma\delta^+$ T cell responses

In the control group proportions and absolute $\gamma\delta^+$ T cells increased 7 to 14 days post infection then decrease 21 to 28 days post infection. However, the initial increase was not significant but the decrease to 0.3 x 10^6/ml 21 days post infection from 0.6 x 10^6/ml 7 days pre-infection was statistically significant ($P<0.05$) (Figure 6.7; Table C3.1 and 3.2 - Appendix IV). Nevertheless, no significant changes were observed in sheep 2381

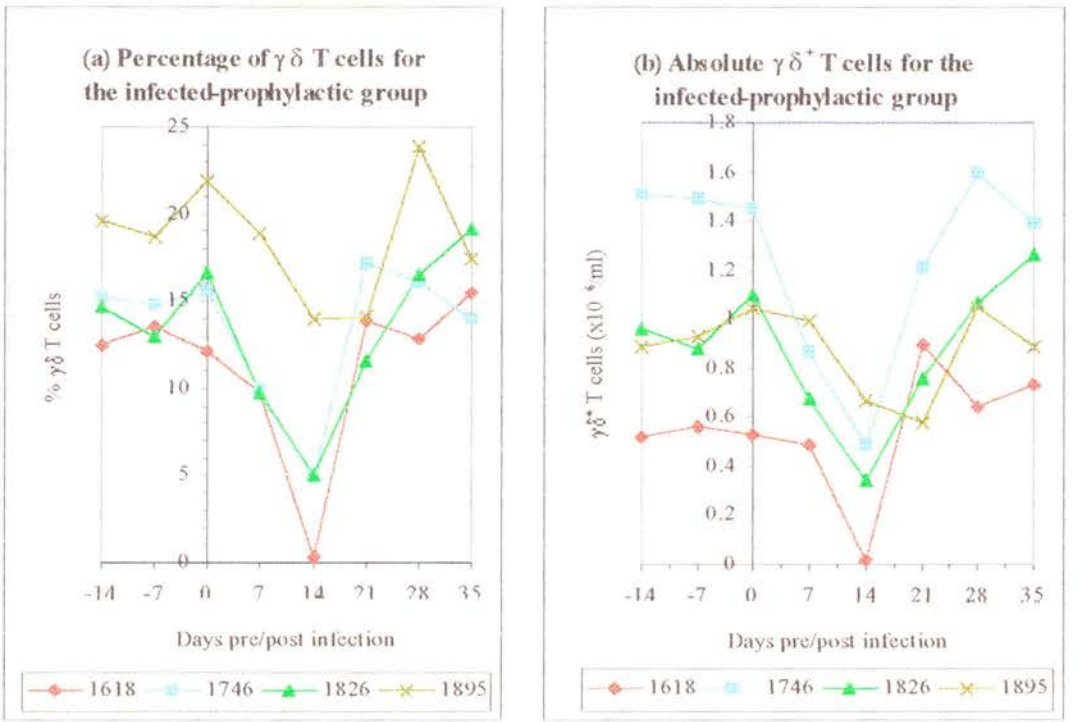
Figure 6.7 $\gamma\delta^+$ T cell responses in the control group



- All sheep were infected with *T. congolense* on day zero

In the prophylactic group the percentage of $\gamma\delta^+$ T cells decreased 14 days after treatment and the median absolute number of $\gamma\delta^+$ T cells was 0.96 $x10^6$/ml 7 days before infection and decreased to 0.38 $x10^6$/ml 14 days post infection (Figure 6.8; Table C3.1 and 3.2 - Appendix IV). But because of great variation when compared to pre-infection values, the decrease was not statistically significance (P>0.05).

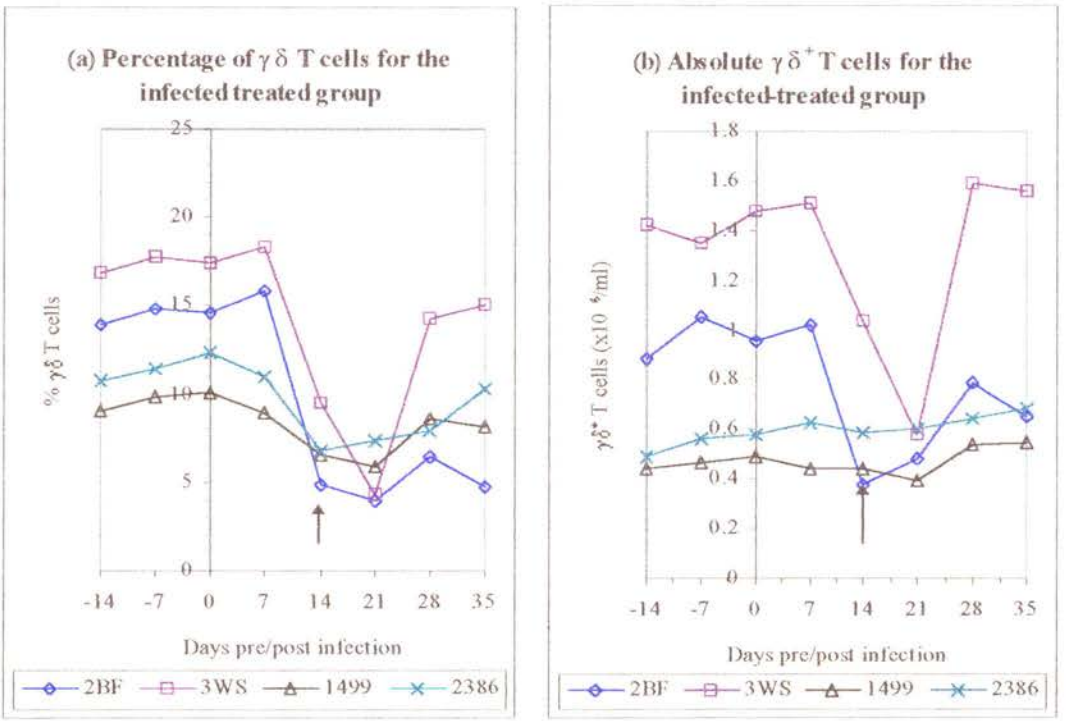
Figure 6.8 $\gamma\delta^+$ T cell responses in the prophylactic group



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered $4^1/_2$ months before infection

In the treated group the percentage of $\gamma\delta^+$ T cells decreased in all sheep 14 - 21 days after infection. However, only 2BF and 3WS showed a significant decrease of absolute $\gamma\delta^+$ T cells 14 to 21 after infection (Figure 6.9; Table C3.1 and 3.2 - Appendix IV). This resulted in a non significant difference ($P>0.05$) between 7 days pre-infection (0.86 $x10^6$/ml) and 21 days post infection (0.51 $x10^6$/ml). Treatment with ISMM 14 days after infection had no effect on $\gamma\delta^+$ T cells.

Figure 6.9 $\gamma\delta^+$ T cell responses in the treated group

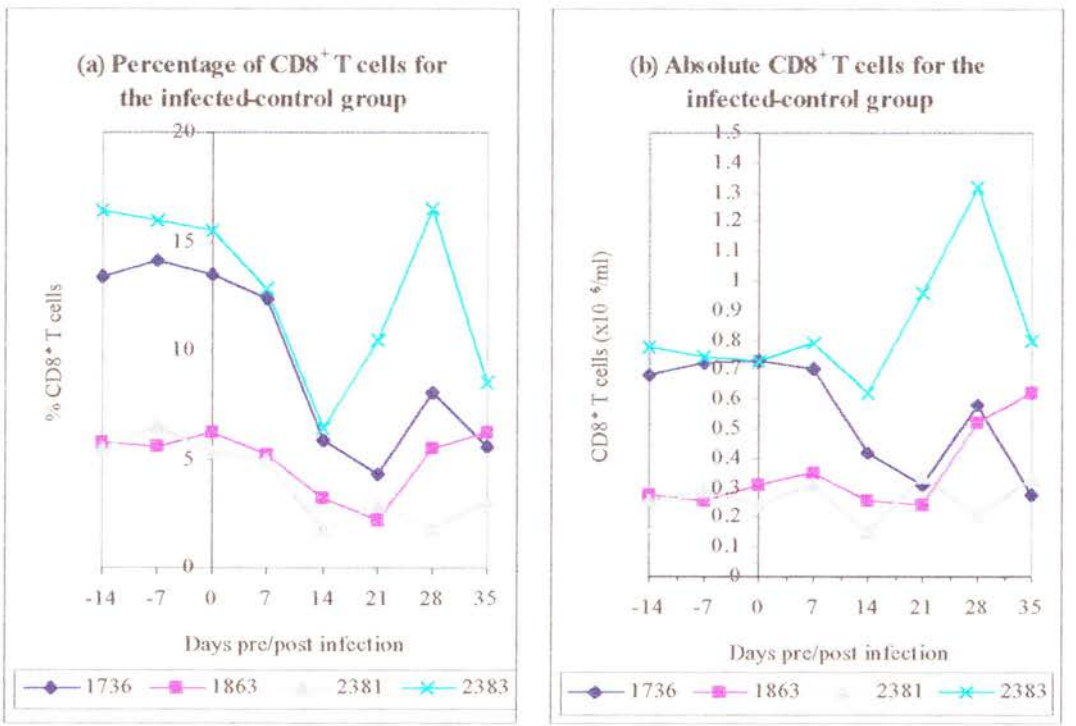


- All sheep were infected with *T. congolense* on day zero
- All sheep were treated with ISMM 14 days after infection (arrow)

6.3.5 $CD8^+$ T cell responses

In the control group the proportions of $CD8^+$ T cells decreased in all sheep 14 - 21 days after infection. However, absolute numbers showed a decrease 14 - 21 days after infection only in sheep 1736 and 1863 (Figure 6.10; Table C4.1 and 4.2 - Appendix IV). No significant changes were observed in sheep 2381 while sheep 2383 showed an increase 21 to 28 days after infection. Results varied greatly and there was no statistical significance between pre-infection and post infection values ($P>0.05$.

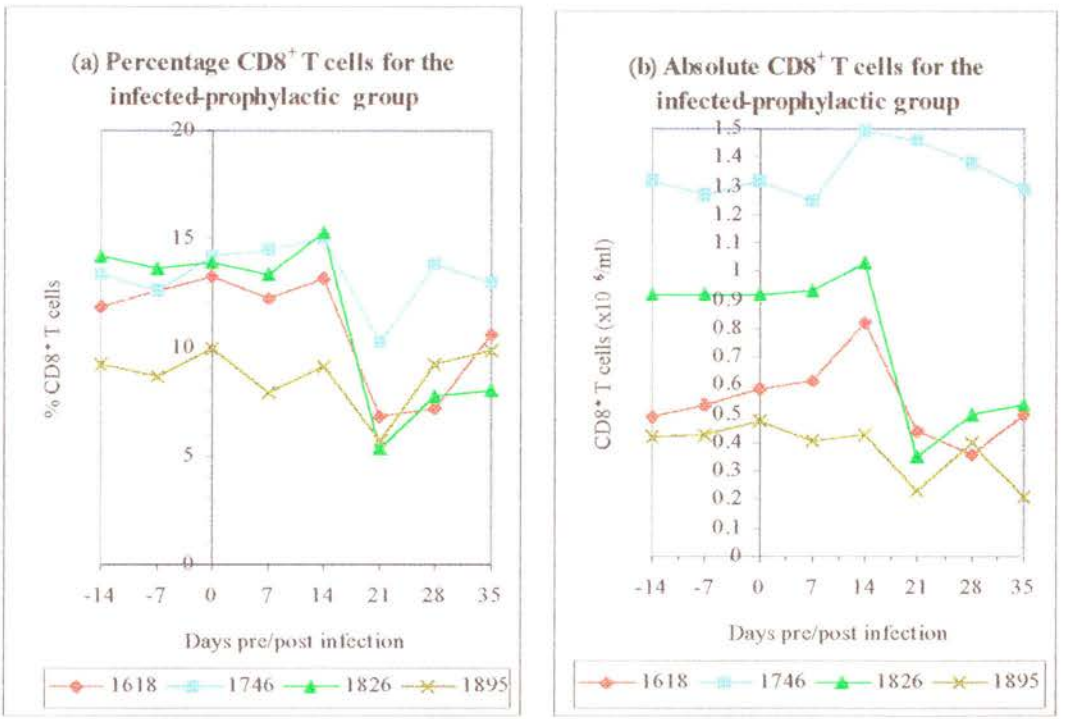
Figure 6.10 $CD8^+$ T cell responses in the control group



- All sheep were infected with *T. congolense* on day zero

In the prophylactic group, the percentage of $CD8^+$ T cells decreased 21 days post infection. Absolute $CD8^+$ T cells decreased significantly 21 days onwards for sheep 1618 and 1826 while sheep 1746 showed a slight increase 14 - 21 days after infection and no changes were observed in sheep 1895 (Figure 6.11; Table C4.1 and 4.2 - Appendix IV).

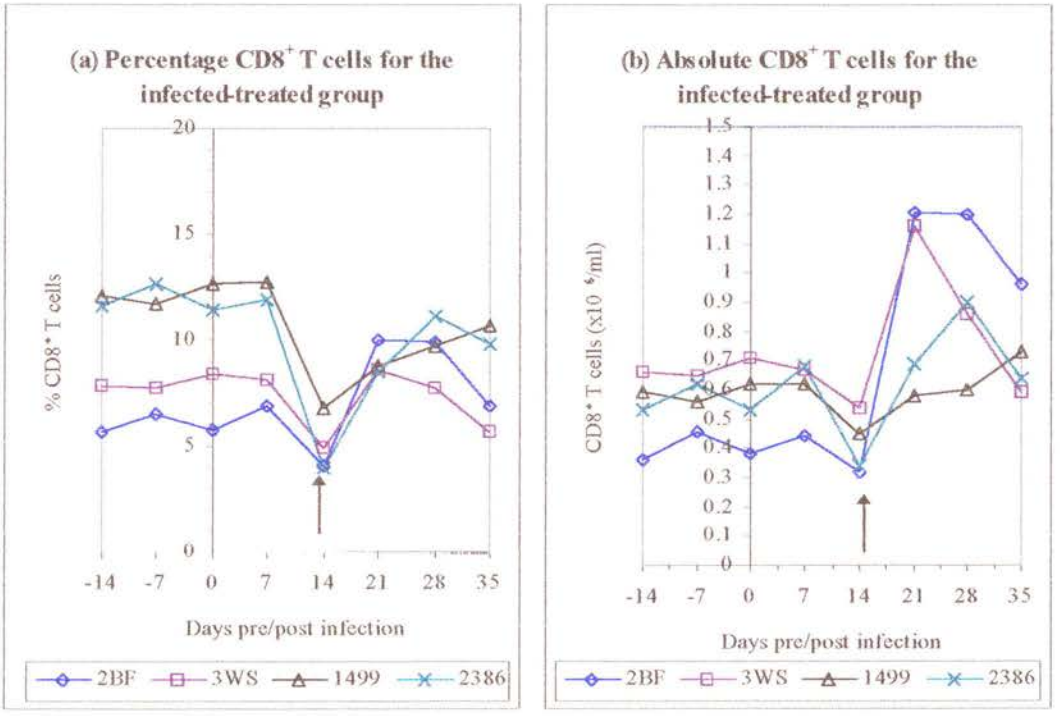
Figure 6.11 $CD8^+$ T cell responses in the prophylactic group



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered $4^1/_2$ months before infection

The treated group showed a decrease in the proportions of $CD8^+$ T cell 14 days after infection. However, absolute $CD8^+$ T cell counts showed that levels were normal until after treatment when a significant ($P>0.05$) increase was observed 21 days onwards. The highest increase in $CD8^+$ T cell numbers was observed in sheep 2BF and 3WS, followed by sheep 2386, while little or no changes were observed in sheep 1499 (Figure 6.12; Table C4.1 and 4.2 - Appendix IV).

Figure 6.12 $CD8^+$ T cell responses in the treated group

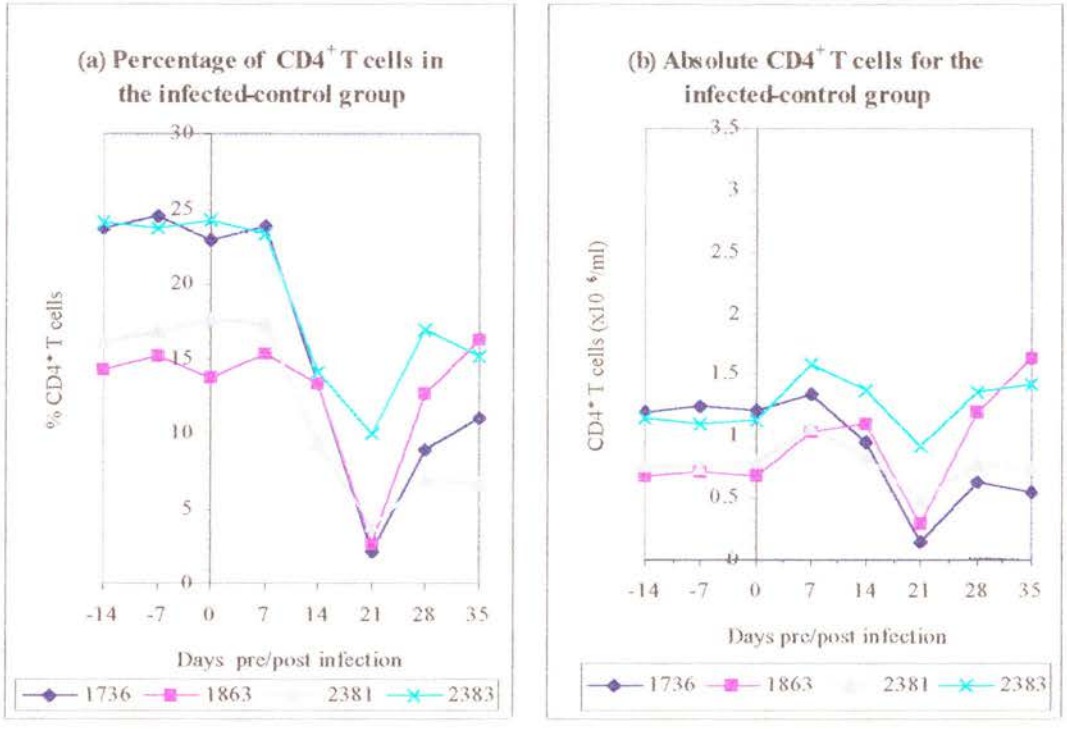


- All sheep were infected with *T. congolense* on day zero
- All sheep were treated with ISMM 14 days after infection (arrow)

6.3.6 $CD4^+$ T cell responses

The control group showed a decrease in the percentage of $CD4^+$ T cells from 21 days onwards in all sheep except sheep which showed only a transient decrease 21 days after infection (Figure 6.13; Table C5.1 and 5.2 - Appendix IV). However, absolute numbers of $CD4^+$ T cells increased non-significantly 7 to 14 days post infection and decreased below normal 21 days post infection (P.0.05).

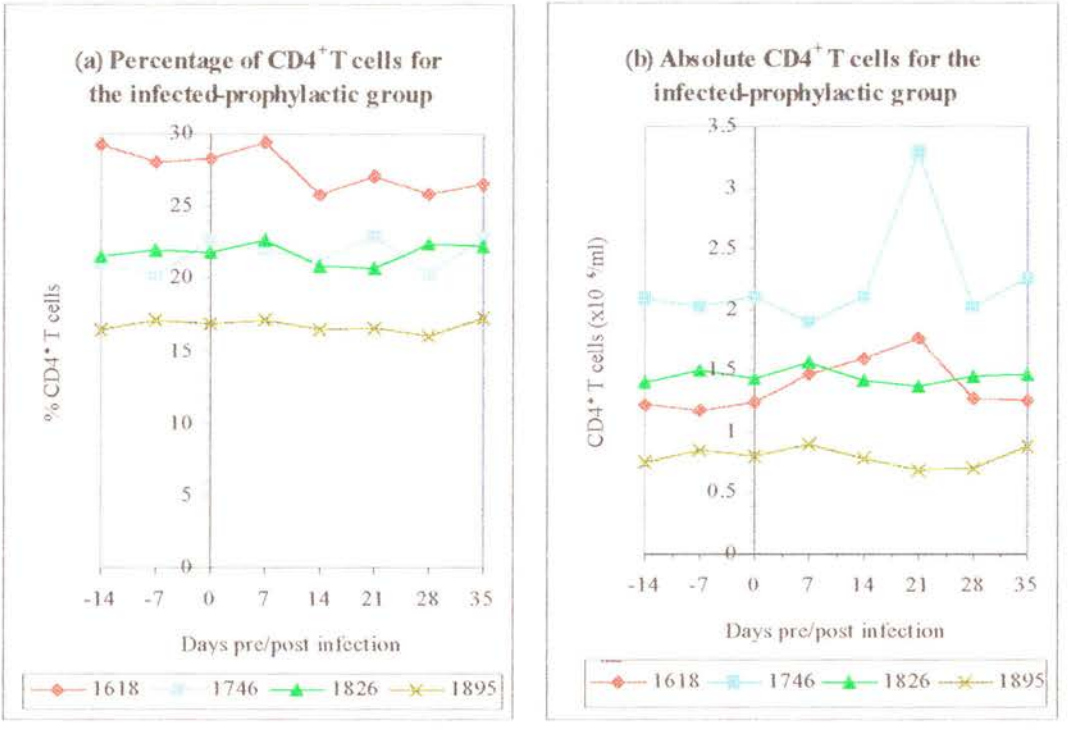
Figure 6.13 $CD4^+$ T cell responses in the control group



- All sheep were infected with *T. congolense* on day zero

In the prophylactic group there was no change in the proportions of $CD4^+$ T cells throughout the experimental period. Nevertheless, absolute numbers for sheep 1746 and 1618 showed a slight increase 21 days post infection (Figure 6.14; Table C5.1 and 5.2 - Appendix IV).

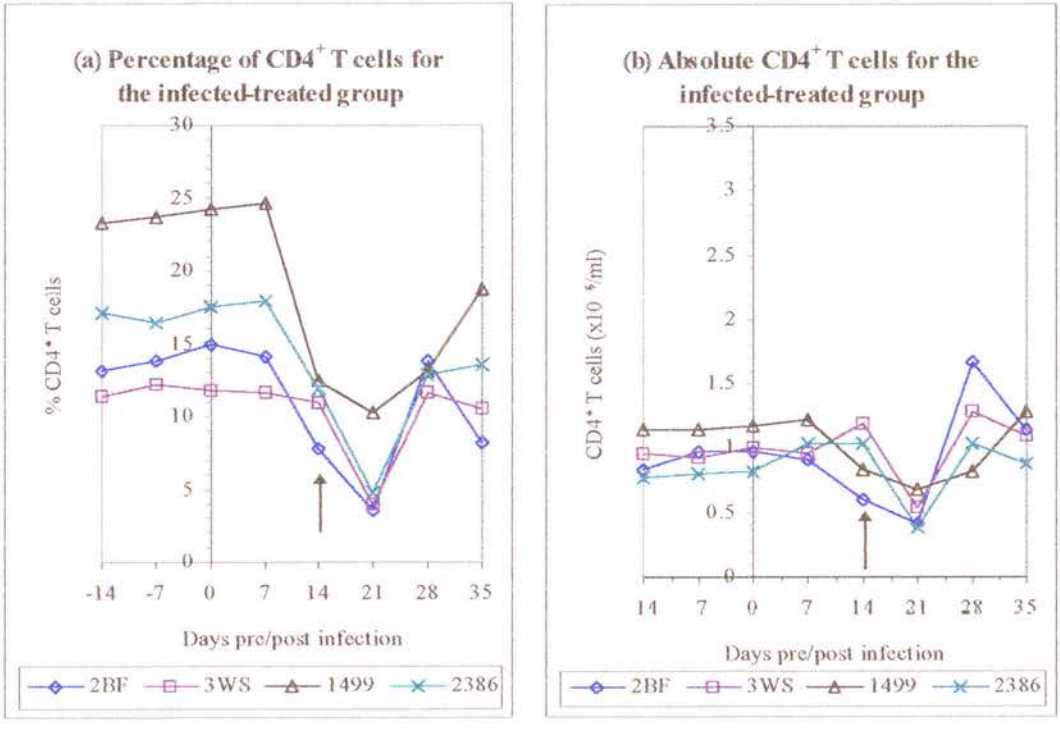
Figure 6.14 $CD4^+$ T cell responses in the prophylactic group



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered $4^1/_2$ months before infection

In the treated group a significant ($P < 0.01$) decrease of both percentage and absolute numbers of $CD4^+$ T cells was observed 21 days post infection in all the sheep (Figure 6.15; Table C5.1 and 5.2 - Appendix IV). Treatment with ISMM 14 days after infection did not change the trend of response since, like in the control, levels returned to normal by 28 days after infection.

Figure 6.15 $CD4^+$ T cell responses in the treated group

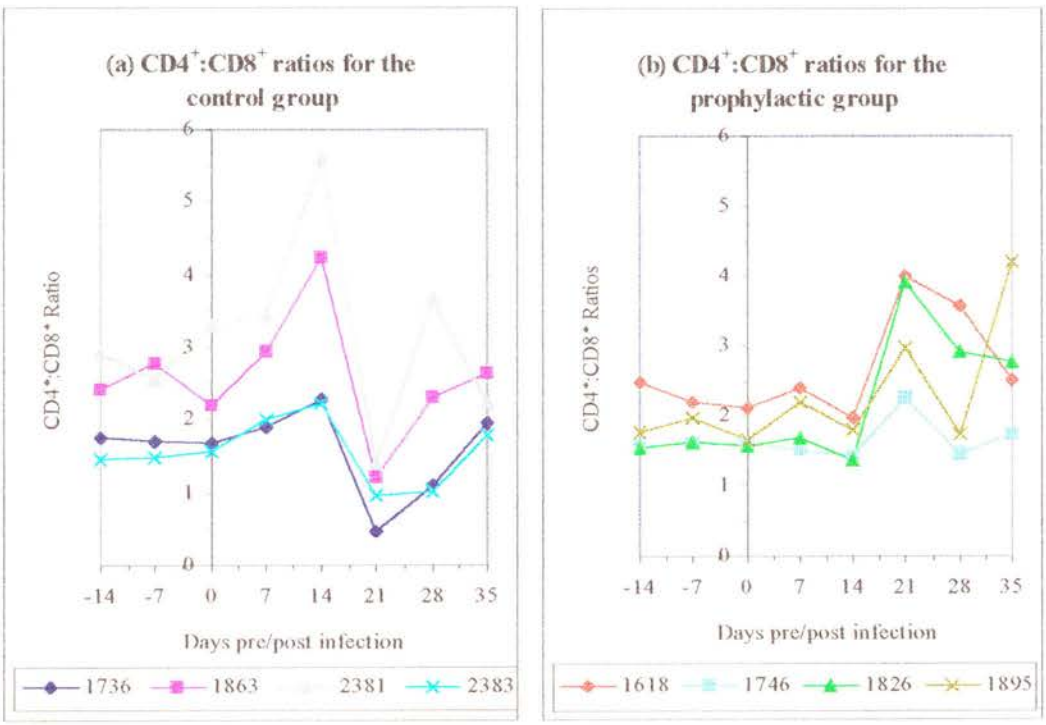


- All sheep were infected with *T. congolense* on day zero
- All sheep were treated with ISMM 14 days after infection (arrow)

6.3.7 $CD4^+$:$CD8^+$ T cell Ratio

Figure 6.16 and Table C6.0 - Appendix IV, shows that the ratio of $CD4^+$:$CD8^+$ T cells increased non significantly 7 to 14 days, then decreased significantly on day 21 in the control ($P<0.05$). In the prophylactic group the ratio increased significantly from day 21 onwards ($P<0.05$). The ratio in the prophylactic 21 days after infection was the opposite of that observed in the control group.

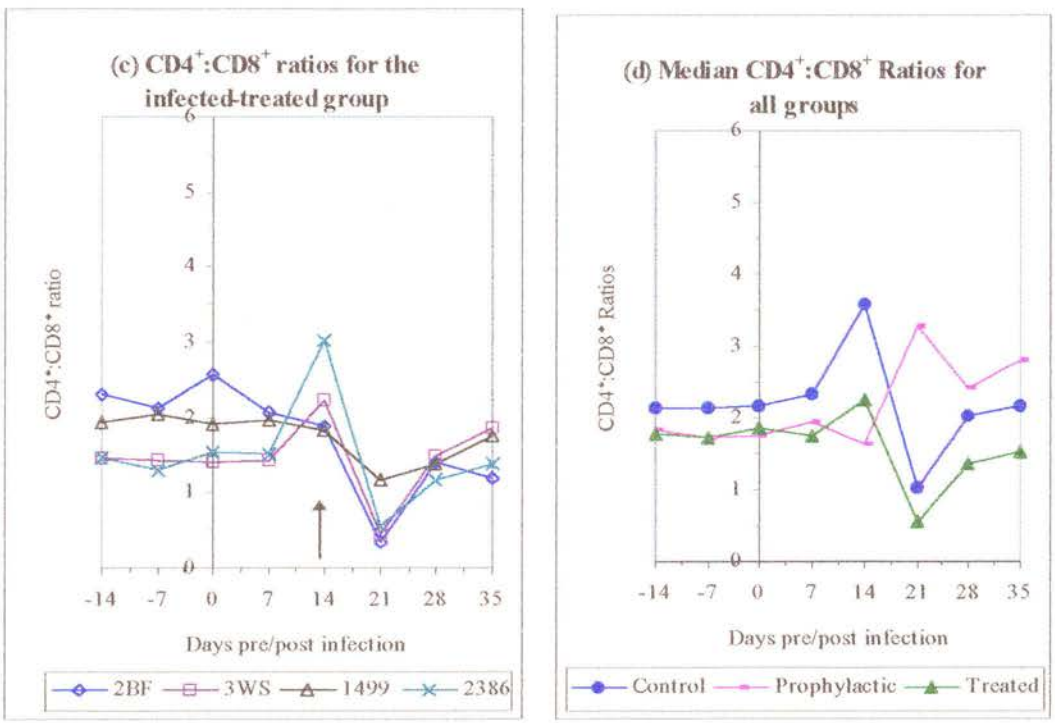
Figure 6.16 $CD4^+$:$CD8^+$ T cell Ratio in the control and prophylactic groups



- All sheep were infected with *T. congolense* on day zero
- ISMM was administered in the prophylactic group $4^1/_2$ months before infection

Figure 6.17(a) and Table C6.0 - Appendix IV shows that the ratio of $CD4^+$:$CD8^+$ T cells in the treated group decreased significantly 21 days post infection ($P<0.01$. A small non-significant increase was observed 14 days post infection. Figure 6.17(b) and Table C6.0 - Appendix IV shows the median ratios of $CD4^+$:$CD8^+$ T cells for all the experimental groups and indicates that the ratio increased in the prophylactic group, while it decreased in the control and treated groups 21 days after infection.

Figure 6.17 $CD4^+$:$CD8^+$ T cell Ratio in the treated group and median values for all the groups

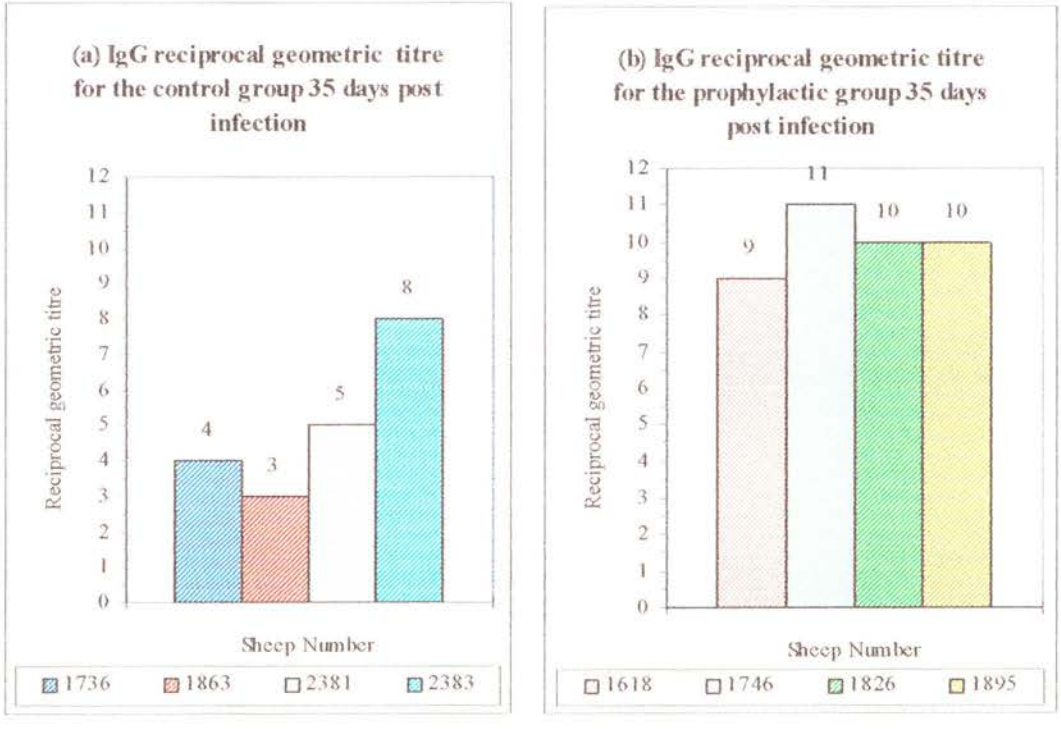


- All sheep were infected with *T. congolense* on day zero
- Prophylactic group: ISMM was administered in the $4^1/_2$ months before infection
- Treated group: All sheep were with ISMM 14 days after infection

6.3.8 Serum IgG antibody response

Trypanosome specific IgG antibodies were measured 35 days post infection for all groups. The antibody titre was determined which was the highest dilution that gave a positive result after subtracting readings for the negative serum. Since a double dilution was carried out on all samples, the median reciprocal antibody titre was converted to geometric median titres (GMT) by finding the antilogarithm base two of the reciprocal titres. The reciprocal titres in the control group ranged from 3 - 8 with a median value of 5 (Figure 6.18(a)). In the prophylactic group the median reciprocal geometric titre was 10 and the ranging from 9 - 11 (Figure 6.18(a)).

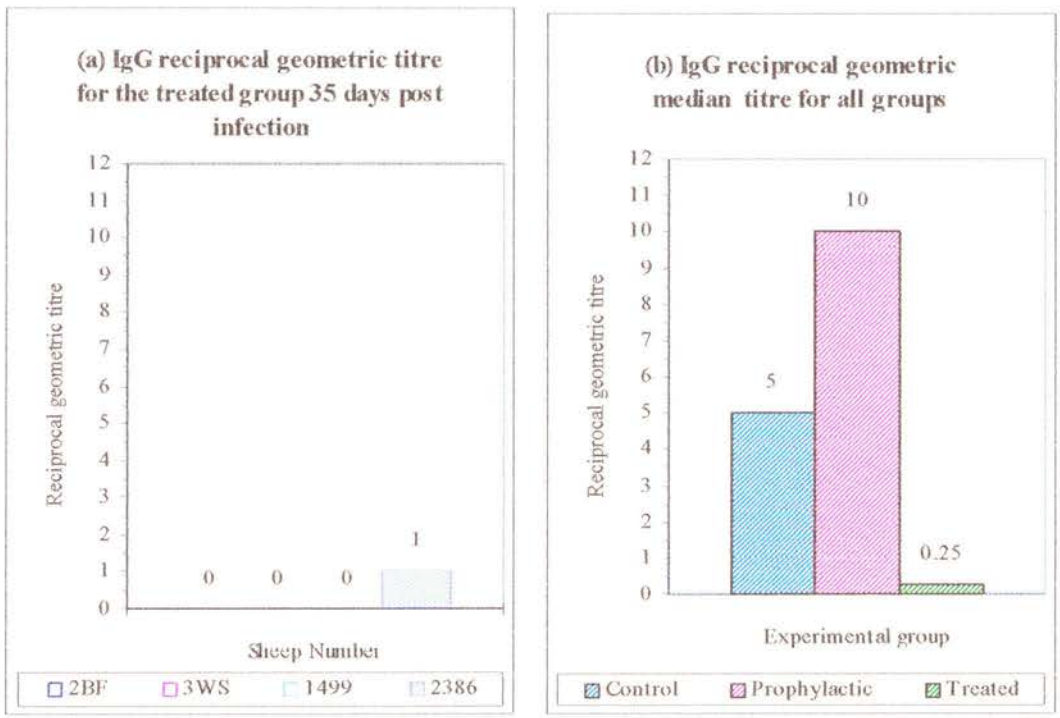
Figure 6.18 Serum IgG antibody response in control and prophylactic groups 35 days after infection



- All sheep were infected with *T. congolense* on day zero
- Prophylactic group: ISMM was administered in the $4^{1}/_{2}$ months before infection

Figure 6.19(a) shows that trypanosome specific IgG antibody levels in all treated sheep were undetectable (reciprocal geometric median titre of 0.25). Figure 6.19(b) summarises IgG responses showing that the difference between the median for the control and the treated groups was statistically significant (P>0.05), and that between the control and the prophylactic groups was also significant (P<0.05) indicating that the prophylactic group had higher antibody levels than the control and treated groups.

Figure 6.19 Serum IgG response in the treated group and median values for the control, prophylactic and treated groups



- All sheep were infected with *T. congolense* on day zero
- Prophylactic group: ISMM was administered in the $4^{1}/_{2}$ months before infection
- Treated group: All sheep were with ISMM 14 days after infection

6.4 DISCUSSION

Results of these experiments indicate that all animals in the control group showed a significant increase in the percentage and absolute number of B cells 14 - 28 days after *T. congolense* infection peaking on day 21. Absolute number of B cells in the prophylactic group did not show any significant increase following *T. congolense* infection. However, sheep number 1746 and 1618 showed a small increase 14 - 21 days after infection, while no detectable changes were observed for sheep 1826 and 1895. In the treated group absolute B cell counts increased significantly from 14 days after infection onwards. Treatment with ISMM 14 days after infection prevented any further increases in sheep 1499 and 2386 while in sheep 2BF and 3WS B cell numbers continued to increase in the same way as in the control group. However, by day 35 B cell numbers had returned to normal levels. Increases in B cell responses have been demonstrated in sheep infected with *T. congolense* (Mwangi 1991) and *T. evansi* (Onah 1990) and in cattle infected with *T. congolense* (Williams *et al* 1991; Lutje *et al* 1995). This study demonstrates that prophylactic administration prevents polyclonal B cell proliferation and that treating an infection 14 days afterwards interferes with B cell proliferation and probably differentiation that would result in the interference with antibody production.

Absolute numbers of $CD5^-$ T cells increased transiently 7 days after infection in the control group. However, that increase was not statistically significant. Sheep in the prophylactic group did not show changes in absolute $CD5^-$ T cells after infection, except sheep 1746 which showed an increase 21 days post infection. None of the sheep in the treated group showed any significant changes in absolute $CD5^+$ T cells through out the experimental period although sheep 2BF exhibited a slight transient increase 28 days after infection. A decrease is proportions of $CD5^+$ T cells were observed only in susceptible animals (Onah 1990; Williams *et al* 1991; Lutje *et al* 1995) not in those that were resistant (Onah 1990; Williams *et al* 1991). Similar results were obtained in this study, where the control and the treated groups showed a decrease in proportions of $CD5^-$ T cells while the prophylactic group did not.

However, absolute numbers of $CD5^+$ T cells in the control showed a sight increase followed by a decrease and not in the treated and prophylactic groups.

Absolute $\gamma\delta^+$ T cells in the control group increased 7 - 14 days after infection then decrease 21 - 28 days post infection. However, the initial increase was not significant but the decrease 21 days post infection was statistically significant. However, no significant changes were observed in sheep 2381. In the prophylactic group, absolute of $\gamma\delta^+$ T cell counts decreased 14 days after infection. In the treated group only 2BF and 3WS showed a significant decrease in absolute $\gamma\delta^+$ T cell counts 14 - 21 after infection. As a result the median value 14 - 21 days after infection was not significantly different from pre-infection levels. Treatment with ISMM 14 days after infection had no significant effect on $\gamma\delta^+$ T cells. Other experiments in trypanosome infected sheep and cattle have demonstrated decreases in $\gamma\delta^+$ T cell phenotypes (Mwangi 1991; Williams *et al* 1991; Lutje *et al* 1995). The role of $\gamma\delta^+$ T cells in trypanosome infections is not well understood but Flynn and Sileghem 1991 demonstrated that only $\gamma\delta^+$ T cells from *T. congolense* infected trypanotolerant N'dama and not susceptible Boran cattle proliferated *in vitro* suggesting that they may be involved in the control of the infection. In this study a decrease in absolute numbers of $\gamma\delta^+$ T cells in the control group occurred 21 days after infection and 14 days after infection in the prophylactic group while only two sheep in the treated group showed a decrease 21 days after infection. However, it is not known whether earlier changes in the prophylactic group have any significance in the control of the infection.

Absolute numbers of $CD8^+$ T cells in the control group showed a decrease 14 - 21 days after infection only in sheep 1736 and 1863. No significant changes were observed in sheep 2381 while sheep 2383 showed an increase 21 to 28 days after infection. Responses varied greatly and there was no statistical significance between pre-infection and post infection values. In the prophylactic group, absolute $CD8^+$ T cells decreased significantly 21 days onwards for sheep 1618 and 1826 while sheep 1746 showed only a slight increase 14 - 21 days after infection and no changes were

observed in sheep 1895. Absolute $CD8^+$ T cell counts in the treated group reviewed that levels were normal until after treatment when a significant increase was observed 21 days onwards. $CD8^+$ T cells have been reported to decrease in both resistant (Onah 1990; Williams *et al* 1991) and susceptible animals (Onah 1990; Mwangi 1991; Williams *et al* 1991; Lutje *et al* 1995) following an infection with trypanosomes. Similar results were obtained in this experiment with decreases in $CD8^+$ T cells being observed in the control and prophylactic groups except in the treated group where a significant increase was observed after treatment with ISMM.

Absolute numbers of $CD4^+$ T cells in the control group showed a non-significant increase 7 to 14 days after infection, then decreased non-significantly below normal 21 days post infection. Changes were not significant, only because of wide variations between individual sheep. In the prophylactic group absolute numbers of $CD4^+$ T cells for only sheep 1746 and 1618 showed a slight increase 21 days post infection. In the treated group a significant decrease in absolute numbers of $CD4^+$ T cells was observed 21 days post infection in all the sheep. Treatment with ISMM 14 days after infection did not change the trend of response since, like in the control, levels returned to normal by 28 days after infection. In general trypanosomes caused a severe loss of $CD4^+$ T cells in susceptible animals (Mwangi 1991; Onah 1992; Lutje *et al* 1995). That loss could have been attributed to TCR-induced apoptosis or programmed cell death (DosReis, Fonseca and Lopes 1995; Fresno, Kopf and Rivas 1997). Activation induced $CD4^+$ T cell death was not observed in animals under ISMM prophylaxis probably via Th_1-type cytokines. Th_1 type cytokines have been shown to block whereas Th_2-type cytokines can enhance TCR-induced programmed cell death (Fresno, Kopf and Rivas 1997).

The ratio of $CD4^+$:$CD8^+$ T cells increased non significantly 7 to 14 days, then decreased significantly on day 21 in the control group, while in the prophylactic group the ratio increased significantly from day 21 onwards. The ratio of $CD4^+$:$CD8^+$ T cells in the treated group decreased significantly 21 days post infection. A small non-significant increase was observed 14 days post infection. The median ratios of

$CD4^+$:$CD8^+$ T cells for all the experimental groups indicate that the ratio increased in the prophylactic group, while it decreased in the control and treated groups 21 days after infection. These results indicate that prophylactic administration of ISMM results in a increase in the ratio of $CD4^+$:$CD8^+$ T cells while treating an established infection 14 days after infection does not change the decrease in the ratio that is observed in the control group. The ratio of $CD4^+$:$CD8^+$ cells in the blood may be used to estimate lymphocyte function in clinical situation. An elevated $CD4^+$ count implies increased lymphocyte reactivity as helper cells predominate, where as a high $CD8^+$ level implies depressed lymphocyte reactivity as a result of excessive suppressor activity (Tizard 1992).

The reciprocal geometric titres of trypanosome specific IgG antibodies in the control group ranged from 3 - 8 with a median value of 5. In the prophylactic group the median reciprocal geometric titre was 10 and the ranging from 9 - 11. However, in the treated antibody levels were very low or undetectable (median titre of 0.25). The difference between the control and the treated groups was statistically significant and that between the control and the prophylactic groups was also statistically significant and the prophylactic group had higher antibody levels than the control and treated groups. Therefore, prophylactic administration of ISMM resulted in a significant increase in levels of trypanosome specific IgG antibodies while treating an established infection 14 days after infection depressed antibody production when compared to the control group. These observations correlates well with changes in the ratio of $CD4^+$:$CD8^+$ T cells and absolute $CD8^+$ T cell counts. Although the pattern of changes in the ratio of $CD4^+$:$CD8^+$ T cells in the control and treated groups were similar, absolute $CD8^+$ T cell counts in the treated group increased significantly after drug treatment, therefore resulting in the depression of antibody production. On the other hand, the increase in the ratio of $CD4^+$:$CD8^+$ T cells in the prophylactic group favoured an increase in helper cell ($CD4^+$) activity resulting in high antibody levels.

Antibody responses in both natural and experimental trypanosome infections have been studied extensively. Infection of cattle with *T. congolense* or *T. vivax* (Luckins

1976) and sheep with *T. congolense*, *T. vivax* or *T. brucei* (MacKenzie, Boyt and Nesham 1979) resulted in increases in serum trypanosome non-specific IgM and IgG antibodies during the course of the infection. Similar increases in trypanosome specific IgM and IgG antibodies following *T. evansi* infection of sheep were reported by Onah (1992). Very few studies have compared patterns of antibody response in susceptible and resistant animals. Results from Onah (1992) showed that sheep that self-cured had higher levels of IgM and IgG antibodies than those that did not self-cure. Suppression of trypanolytic antibodies in an ISMM prophylactic (Whitelaw *et al* 1986; Geerts *et al* 1997) and treated (Whitelaw *et al* 1986) groups of cattle has been demonstrated. In this study trypanosome specific IgG antibodies were down-regulated in the treated group and up-regulated in the prophylactic group. Probably the difference in relation to prophylactic group is a result of antibody assays used since in this study antibody ELISA was used while Whitelaw *et al* (1986) and Geerts *et al* (1997) used the trypanolytic assay. However, results from treated animals are similar, implying that trypanolytic antibodies may not be of the IgG isotype, therefore they might be of the IgM isotype. These observation emphasise the fact that during trypanosome infection both parasite specific and non-specific antibody response may decrease, and that ISMM administration can lead to down-regulation or up-regulation of certain antibody isotype responses.

These results demonstrate that there are significant differences in immunological responses between animals under ISMM prophylaxis and control or infected and treated animals. ISMM prophylaxis prevented polyclonal B cell or T cell proliferation probably in favour of clonal proliferation. It also prevented significant losses of $CD4^+$ T cells while not effecting $CD8^+$ T cell reduction resulting in an increased helper T cell activity and a high trypanosome specific IgG antibody response. However, treatment of an infection with ISMM 14 days afterwards interfered with ongoing immune responses resulting in an inhibition of trypanosome specific IgG antibody responses.

In conclusion, ISMM prophylaxis prevented *T. congolense* infection and modified cellular immunological responses resulting in high IgG antibodies. On the other hand

treatment of the infection suppressed IgG antibody response. These differences indicates that the mechanism of modulation involves cytokines which could either up-regulate or down-regulate antibody production depending on the status of the immune system at the time of infection or ISMM administration repectively.

CHAPTER 7

CHAPTER 7
MONONUCLEAR CELL PROLIFERATION AND CYTOKINE PRODUCTION

7.1 INTRODUCTION

This chapter describes results of the effect of ISMM on IFN-γ production and proliferative responses of sheep peripheral blood mononuclear cells as well as IL-12 and IFN-γ production by mouse splenic cells. Flynn *et al* (1991) reported a depression of *in vitro* proliferative responses of peripheral blood lymphocytes and lymph node cells from cattle infected with *T. congolense* when stimulated with T cell mitogen concanavalin A (ConA). On the other hand, no proliferative responses to purified trypanosome antigen specific stimulation were detected in peripheral blood lymphocytes before infection, at any point after infection or treatment in cattle. However, proliferation was observed in draining lymph node cells (Lutje *et al* 1995).

IL-12 has been reported to play a strong regulatory role on IFNγ production in many infections such as *Histoplasma capsulatum* (Zhou *et al* 1997) and *Bordetella perussis* (Mahon *et al* 1996). IL-12 is rapidly produced after infection by phagocytic cells (monocyte/macrophages and neutrophils (D'Andrea *et al* 1992) and by antigen presenting cells (dendritic cells and skin Langerhans' cells (Macatonia *et al* 1995: Kang *et al* 1996). It elicits the production by T cells and natural killer cells of IFN-γ which activates phagocytic cells. The production of IL-12 is strictly regulated by negative and positive feedback mechanisms. If IL-12 and IL-12 induced IFN-γ are present during early T cell expansion in response to antigen, Th_1 cell generation is favoured and the generation of Th_2 cells inhibited. Thus IL-12 is a potent immunoregulatory cytokine which promotes Th_1 resistance to intracellular parasite infection (Trinchieri 1997). Both Th_1 and Th_2 responses have been reported in trypanosome infections (Scheifer and Mansfield 1993), but it is not clear which one is associated with pathology or resistance to infection.

7.2 MATERIALS AND METHODS

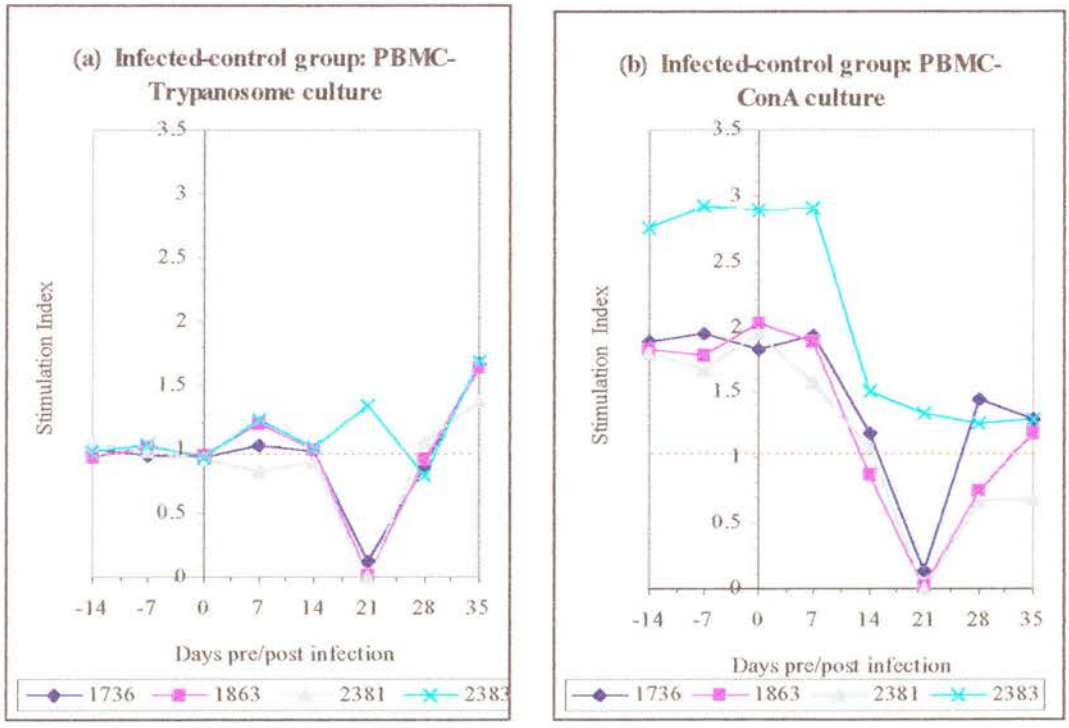
In sheep experiments, PBMCs were collected weekly and cultured in the presence of ConA, live *T. congolense* and media only for 48 hours before collecting the supernatant for the IFN-γ assay. Also PBMCs were cultured *in vitro* in media only, ConA and live *T. congolense* for 72 hours and proliferation determined using the Promega assay and Flow cytometry. Splenic cells were obtained from naive mice and those that had been treated with 1 mg/kg ISMM for 7, 14 and 21 days. They were cultured *in vitro* for 24 hours, after which, IFN-γ and IL-12 were assayed. Details of the protocols and assays are described in Chapter 3: Sections; 3.7, 3.9, 3.10 and 3.11.

7.3 RESULTS

7.3.1 *In vitro* peripheral blood mononuclear cell proliferation

PBMCs were cultured in the presence of trypanosomes and ConA. The proportion of viable cells in these cultures determined using the Promega assay were compared with cultures in media only. A significant decrease in cell viability was observed in trypanosome cultures with PBMCs from control sheep 1736, 1863 and 2381 ($P<0.05$) 21 days post infection but sheep 2383 was not affected. Proliferation in cultures with ConA dropped significantly from day 14 to 35 in the control group (Figure 7.1; Table D1-Appendix V).

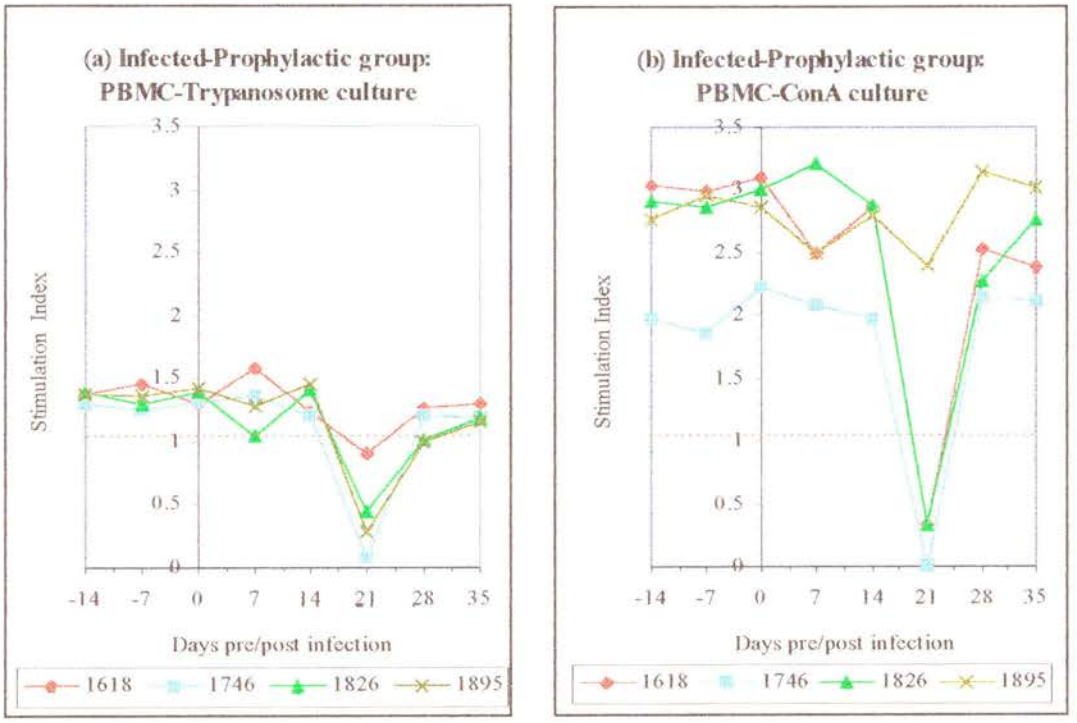
Figure 7.1 *In vitro* peripheral blood mononuclear cell proliferation (Stimulation Index) for the infected Control group



- All sheep were infected on day zero
- Stimulation index of 1 represents normal cell viability in culture (dotted line)
- Stimulation index below 1 indicates accelerated cell death
- Stimulation index above 1 indicates cell proliferation

In the prophylactic group, results of the proliferation assay before infection (i.e. day -14 to 0) were significantly higher than those observed in the control group ($P < 0.01$) ConA induced significant proliferation in PBMCs from all sheep. However, 21 days after infection proliferation in trypanosome cultures fell below normal levels ($P<0.01$). In the ConA cultures, proliferation also decreased below normal ($P<0.05$) 21 days after infection except for sheep 1895 (Figure 7.2; Table D2-Appendix V).

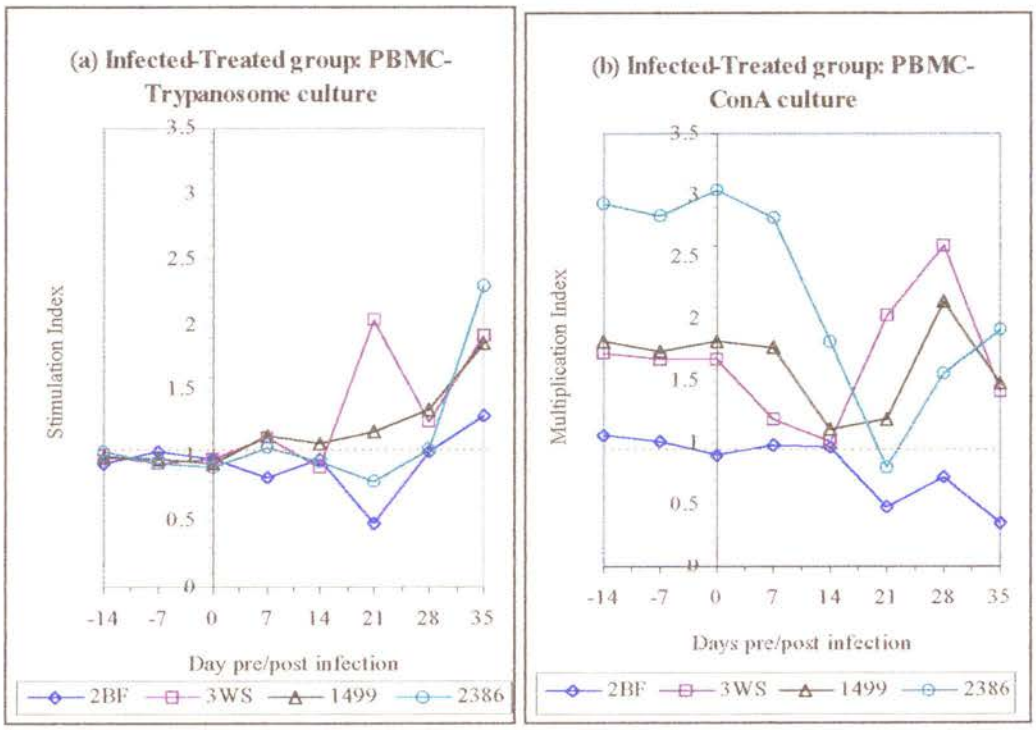
Figure 7.2 *In vitro* peripheral blood mononuclear cell proliferation (Stimulation Index) for the infected Prophylactic group



- All sheep were infected on day zero
- Stimulation index of 1 represents normal cell viability in culture (dotted line)
- Stimulation index below 1 indicates accelerated cell death
- Stimulation index above 1 indicates cell proliferation
- All sheep were prophylactically treated with ISMM $4^1/_2$ months before infection

Proliferation of cells in trypanosome cultures from the treated group 14 to 35 days post infection was significantly ($P<0.05$) higher than normal levels (Figure 7.3; Table D3-Appendix V). Sheep 2BF did not proliferate due to ConA at any time and accelerated cell death was observed 14 days after infection onwards. Sheep 3WS, 1499 and 2386 proliferated due to ConA but a transient non-significant decrease was observed 14 to 21 days after infection.

Figure 7.3 *In vitro* peripheral blood mononuclear cell proliferation (Stimulation Index) for the infected Treated group

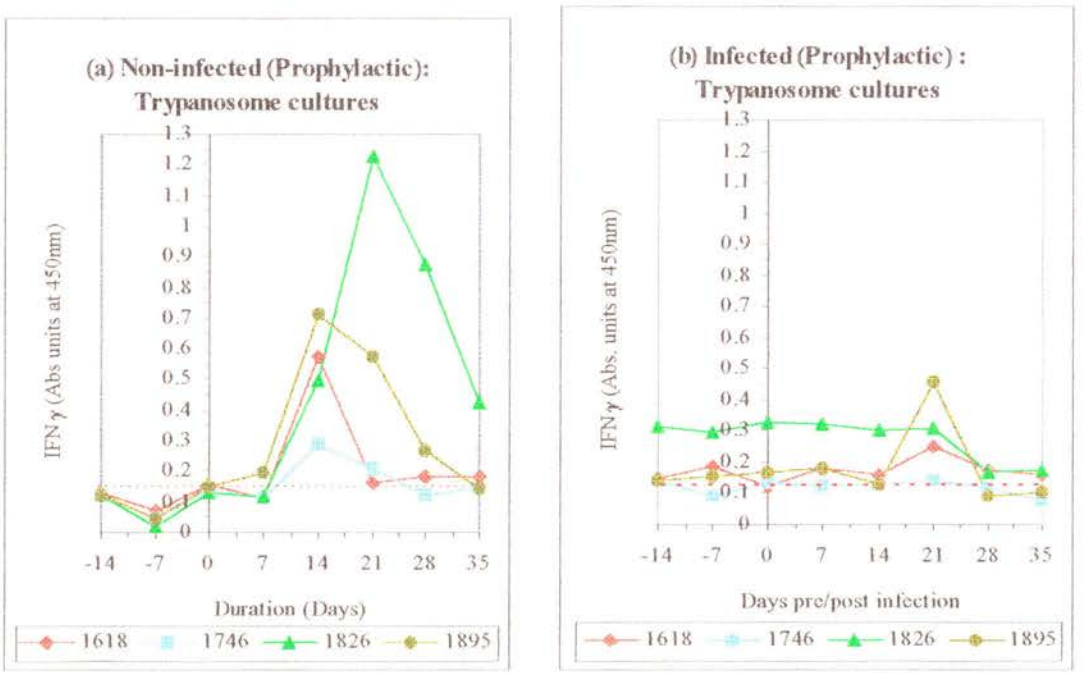


- All sheep were infected on day zero
- Stimulation index of 1 represents normal cell viability in culture (dotted line)
- Stimulation index below 1 indicates accelerated cell death
- Stimulation index above 1 indicates cell proliferation
- All sheep were treated with ISMM 14 days after infection

7.3.2 Supernatant sheep IFN-γ levels from PBMCs in culture

PBMCs from the prophylactic group were cultured with trypanosomes and supernatant IFN-γ levels were determined. Results show that cells from the non-infected prophylactic group cultured in the presence of trypanosomes produced significantly ($P<0.01$) high level of IFN-γ 14 to 28 days after ISMM administration (Figure 7.4a; Table D4-Appendix V). Sheep 1826 continued to produce IFN-γ until 21 days after infection, when it dropped to normal. During the infection period no significant amounts of IFN-γ were detected for the other sheep (Figure 7.4b; Table D4-Appendix V).

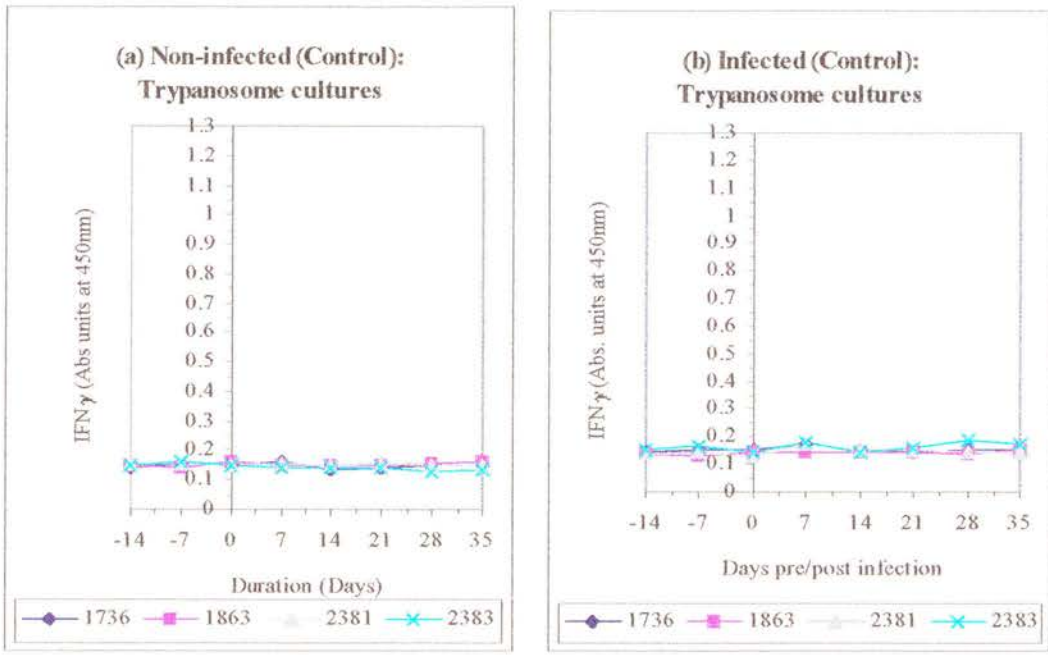
Figure 7.4 Supernatant sheep IFN-γ levels (Absorbance) for Prophylactic PBMCs in culture with trypanosomes



- All sheep in Figure 7.4(a) were injected with ISMMon day 0
- All sheep in Figure 7.4(b) were infected with *T. congolense* on day 0
- Cut-off point was mean of negative control plus twice its standard deviation = 0.163

PBMCs from the control group were also cultured with trypanosomes and supernatant IFN-γ levels were determined. Figure 7.5 and Table D5-Appendix V shows that no IFN-γ was detected in culture supernatants either before or after infection.

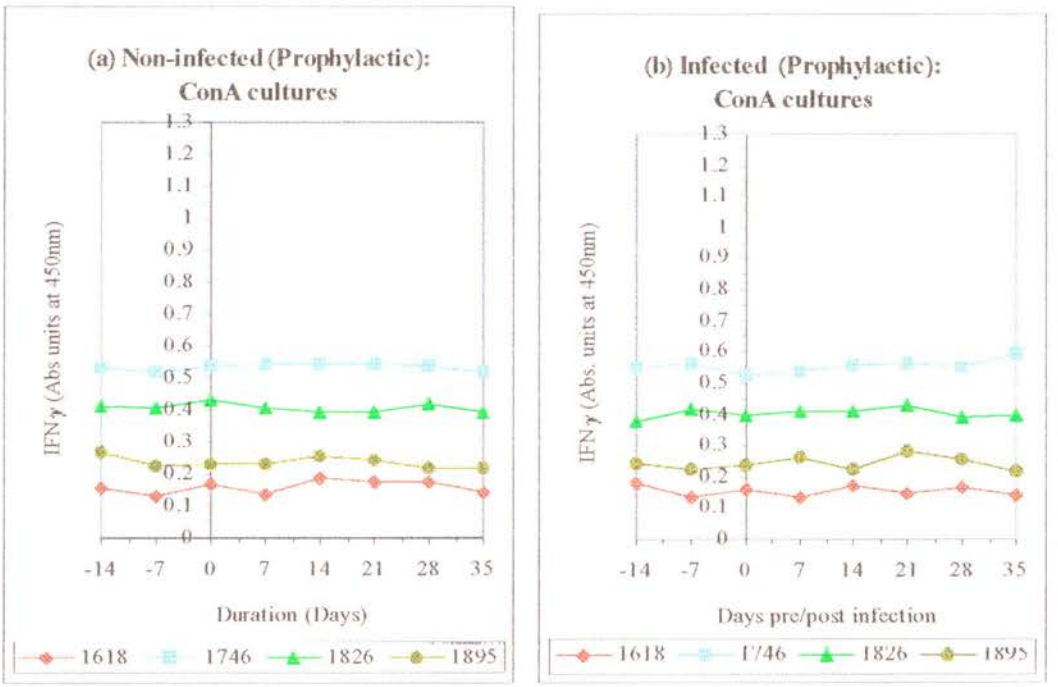
Figure 7.5 Supernatant sheep IFN-γ levels (Absorbance) for Control PBMCs in culture with trypanosomes



- All sheep in Figure 7.5(a) were non-treated and non-infected controls
- All sheep in Figure 7.5(b) were normal and infected with *T. congolense* on day 0
- Cut-off point was mean of negative control plus twice its standard deviation = 0.163

PBMCs in culture with ConA produced significant levels of IFN-γ in non-infected or infected prophylactically treated sheep. ISMM prophylaxis or infection with *T. congolonse* had no effect on IFN-γ production in these cultures (Figure 7.6; Table D6-Appendix V).

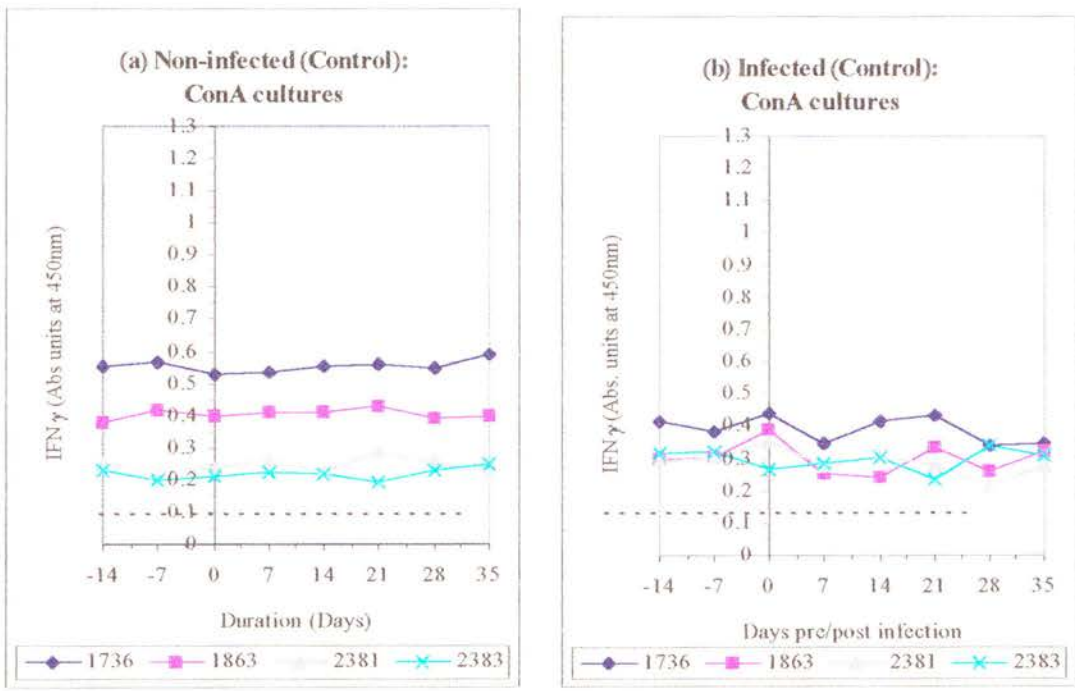
Figure 7.6 Supernatant sheep IFN-γ levels (Absorbance) for Prophylactic PBMCs in culture with ConA



- All sheep in Figure 7.6(a) were injected with ISMM on day 0
- All sheep in Figure 7.6(b) were infected with *T. congolense* on day 0
- Cut-off point was mean of negative control plus twice its standard deviation = 0.163

In the control group IFN-γ production in cultures with ConA produced significant levels of IFN-γ in non-infected or infected sheep. Infection with *T. congolonse* had no effect on IFN-γ production in these cultures as well (Figure 7.7; Table D7-Appendix V).

Figure 7.7 Supernatant sheep IFN-γ levels (Absorbance) for Control PBMCs in culture with ConA

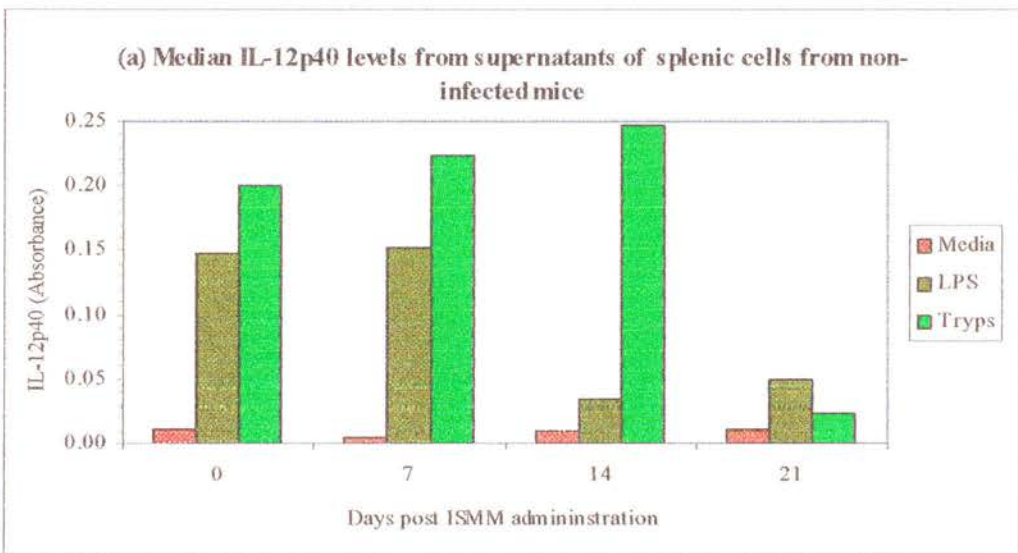


- All sheep in Figure 7.7(a) were non-treated and non-infected controls
- All sheep in Figure 7.7(b) were normal and infected with *T. conglense* on day 0
- Cut-off point was mean of negative control plus twice its standard deviation = 0.163

7.3.3 IL-12 and IFN-γ production from non-infected mice

IL-12p40 production by splenic cell cultures with trypanosomes was high in the non-treated group 7 - 14 days following ISMM administration, but was significantly ($P<0.01$) suppressed in mice that had received ISMM 21 days before in vitro culture (Figure 7.8a; Table D8-Appendix V). Levels of IL-12p40 in cultures with LPS was significantly ($P<0.01$) suppressed in those treated with ISMM 14 and 21 days before culture.

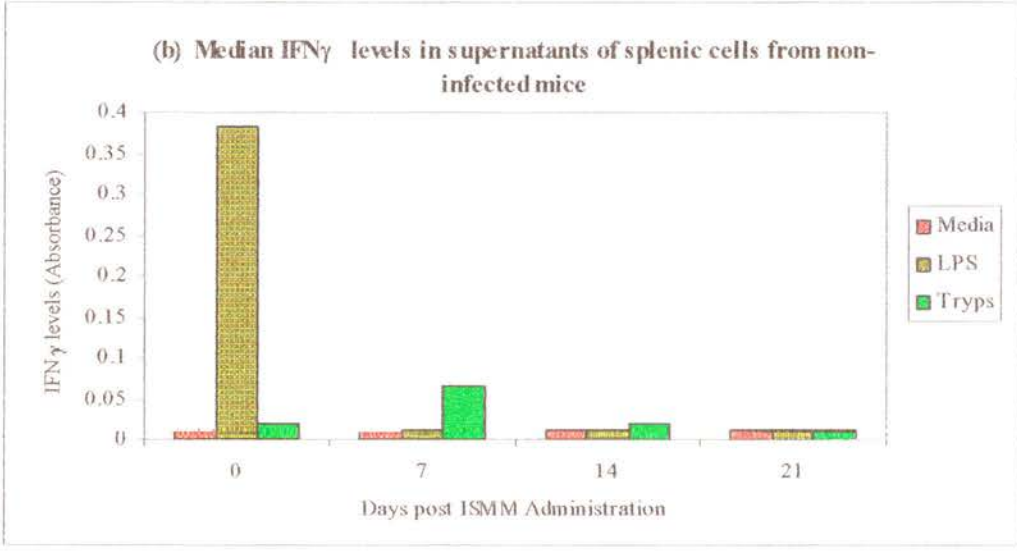
Figure 7.8(a) IL-12 production from non-infected mice



- Day zero represents cells from mice that did not receive ISMM

The same mice splenic culture supernatants in which IL-12 was determined was used for measuring IFN-γ levels. Significant levels of IFN-γ were produced only in non-treated LPS cultures ($P<0.01$). There was no IFN-γ in either ISMM treated or non-treated trypanosome cultures (Figure 7.8b and Table 7.8b).

Figure 7.8 (b) IFN-γ production from non-infected mice



- Day zero represents cells from mice that did not receive ISMM

7.4 DISCUSSION

Proliferation of PBMCs in response to ConA occurred in the control group until 14 days after infection when a decrease was observed. A stimulation index of 1 indicates normal cell viability without proliferation or excessive cell death. Twenty one days after infection, the stimulation index was below normal implying that cell viability in the presence of ConA was at its lowest. However, cell viability returned to normal

thereafter, although proliferative responses to ConA were not restored throughout the study period. In the prophylactic group, only a transient loss of cell viability and proliferative response was observed 21 days post infection. In the treated group changes in proliferative responses to ConA varied greatly, with a decrease occurring between 14 and 21 days. Thereafter, the proliferative responses were restored. These results are similar to those of other workers except their experiments were not related to ISMM prophylaxis. For instance, suppression of proliferative responses to ConA by mononuclear cells isolated from peripheral blood, spleen, lymph nodes has been observed in Boran cattle infected with *T. congolense* at different stages of infection (Flynn and Sileghem 1991). Suppression was greatest in lymph node cells than in spleen cells or PBMCs. Also, Taylor, Lutje and Mertens (1996) using lymph node mononuclear cells in culture with trypanosome lysates demonstrated a significant decrease in proliferative responses to ConA 11 days post infection.

Experiments involving culture of PBMCs with heterologous trypanosomes showed no antigen non-specific proliferative responses in the control group throughout the study period, but a decrease in viability was observed 21 days post infection. The stimulation index for pre-infection PBMCs was significantly higher than 1 implying that they were sensitised to proliferate when stimulated with trypanosomes. However, viability significantly dropped below normal 21 days after infection. Treatment with ISMM 14 days after infection prevented a decrease in cell viability and resulted in an increased proliferative response 21 days onwards. These results indicate that antigen non-specific proliferative responses occurred 21 days after ISMM treatment and could only decrease transiently about 21 days after infection. In addition no antigen non-specific proliferation was observed on the control group. Other studies have shown *in vitro* antigen specific proliferative responses of lymph node mononuclear cells not PBMCs 11 days after infection and reduced responses before or afterwards (Lutje *et al* 1995; Taylor, Lutje and Mertens1996). Gasbarre, Hug and Louis (1981) also demonstrated antigen specific proliferation of mice lymph node cells 2 to 3 weeks after infection with *T. brucei* followed by loss of responsiveness. On the other hand Flynn, Sileghem and Williams (1992) using whole trypanosome lysates obtained the

opposite results where there was proliferation in PBMCs and not in lymph node cells. Purified $CD8^+$ and $\gamma\delta^+$ T cells from peripheral blood of trypanotolerant N'dama cattle undergoing a primary *T. congolense* infection proliferated *in vitro* in response to trypanosome specific invariant but not variant trypanosome antigens 14 to 42 days post infection. No trypanosome antigen specific proliferation was observed in trypanosusceptible Boran cattle (Flynn and Sileghem 1994). It seems proliferation of PBMCs to trypanosome antigens is associated with animals that are relatively resistant to trypanosomiasis. This might explain why researchers that used susceptible Boran cattle were unable to detect PBMC proliferation. In addition it might also explain why ISMM treated sheep in this study showed proliferative responses to trypanosomes 21 days after ISMM treatment onwards.

The loss of antigen non-specific proliferative responses and cell viability observed in this study 21 days after infection coincided with the height lymphocytosis resulting from *in vivo* antigen stimulation. This is in agreement with observations by Taylor, Lutje and Mertens (1996) that an increase in antigen specific proliferative responses coincided with a decrease in proliferation of the same cells in culture with ConA. A decrease of cell numbers below normal (cell death) is likely to be a result of activation induced apoptosis (Kabelitz, Pohl and Pechhold 1993). Apoptosis or programmed cell death is an active, signal-dependent process with characteristic morphological changes in dying cells. Activation renders peripheral T cells susceptible for apoptosis. Truly resting mature T cells are resistant to apoptosis, but gradually become sensitive to programmed cell death after mitogenic or antigenic activation. The sensitivity of activated T cells to apoptosis does not appear to correlate to any distinct phenotype. Thus $CD8^+$, $CD4^+$ and $\gamma\delta$ T cells are all sensitive and within the $CD4^+$ compartment there is no major difference between Th_1 and Th_2 subsets. This suggests that activation induced cell death occurs in $CD4^+$ T cells irrespective of the type of cytokine they produce upon activation. ISMM prophylaxis increased proliferative responses of PBMCs to trypanosome antigens, but had no affect on transient activation induced cell death. On the other hand treating an established infection with

ISMM 14 days post infection prevented activation induced cell death and improved proliferative responses to trypanosome stimulation

IL-12p40 and IL-12p70 were assessed in the same mice splenic cell culture supernatants that were used to assess IFN-γ. None of the cultures were positive for IL-12p70 indicating that it was either not produced or levels were too low to be detected by the assay. On the other hand, IL-12p40 levels were high in trypanosome cultures of non-treated as well as the 7, and 14 day ISMM treatment groups but dropped significantly in the 21 day ISMM treatment group.

At present there are no reports of IL-12 production in trypanosome infections. Nevertheless, studies with other antigens show that its production is induced by bacteria, intracellular pathogens, fungi, viruses or their products by T cell-independent pathway and/or by a T cell-dependent pathway mediated through CD40-CD40 ligand interaction (Trinchieri 1995; Trinchieri 1993 IL-12p70 is rapidly produced after infection by phagocytic cells (monocyte/macrophages and neutrophils (D'Andrea *et al* 1992) and by antigen presenting cells (dendritic cells and skin Langerhans' cells (Macatonia *et al* 1995; Kang *et al* 1996). IL-12p70 is a heterodimeric cytokine composed of 35-kDa(p35) and 40-kDa(p40) subunits. It has been demonstrated that the expression of p35 and p40 genes is differentially regulated by activation of the producing cells. The p40 subunit is responsible for receptor binding and if produced as a monomer or homodimer in the absence of p35 can act as an antagonist to the biological effects of IL-12p70 (Mattner *et al* 1993). Biological effects of IL-12 (p70 heterodimer) include: induction of cytokine production (especially IFN-γ) by T and NK cells, haematopoiesis, enhancement of cell-mediated cytotoxicity and mitogenic effects on T and NK cells.

The production of IL-12 is strictly regulated by negative and positive feedback mechanisms, with IFN-γ being the most potent upregulator (Kubin, Chow and Trinchieri 1994) and IL-10 as the most important down regulator (D'Andrea *et al* 1993). If IL-12p70 and IFN-γ are present during early T cell expansion in response to

antigen, Th_1 cell generation is favoured and the generation of Th_2 cells inhibited. Thus IL-12 is a potent immunoregulatory cytokine which promotes Th_1 resistance to infection (Trinchieri 1997). Both Th_1 and Th_2 responses have been reported in trypanosome infections (Schleifer and Mansfield 1993), but it is not clear which one is associated with pathology or resistance to infection. Thus, in this study the absence of IL-12p70 and presence of high levels of IL-12p40 could have resulted in a down regulation of IFN-γ production in ISMM treated mice splenic cell cultures.

In sheep experiments IFN-γ production in PBMC cultures with ConA was neither related to stage of infection nor ISMM treatment. But the most significant IFN-γ production results in sheep were those produced in the prophylactic group 14 to 21 days after ISMM administration. Sheep 1826 remained sensitised until 21 days after infection. These results indicate that ISMM primed naive PBMCs *in vivo* for high IFN-γ production *in vitro* after challenge with heterologous trypanosome antigens. In the literature there are not reports of IFN-γ production by PBMCs form either naive or trypanosome infected animals when cultured with trypanosome antigens. Results by Lutje *et al* (1995) showed that high levels of IFN-γ were detected from *in vitro* trypanosome antigen culture supernatants of lymph node cells in Boran cattle 11 days after infection with *T. congolense*, but not from trypanosome PBMC culture supernatants. Similar results were obtained by Taylor, Lutje and Mertens (1996) who were able to detect IFN-γ from lymph node cells 11 and 31 days after infection.

Trypanosomes did not induce IFN-γ production in splenocytes from either ISMM treated or un-treated mice. However, LPS induced IFN-γ production only in splenic cells from mice that were not treated with ISMM. These results do not agree with those obtained from studies in *T. brucei* infected mice where IFN-γ production was induced in a non-specific manner (Darji *et al* 1991; Sileghem, Darji and De Baetselier 1991; Olsson *et al* 1991; Bakhiet *et al* 1996). The difference may be due to the fact that, in this study *T. congolense* instead of *T. brucei* was used in cultures. In addition, the pattern of IFN-γ production obtained in this study is different from that in sheep.

In sheep the response increased 14 to 21 days after treatment while in mice, the response was high in naive and was not there in treated mice. This would be beneficial in mice where IFN-γ is associated with pathogenesis and parasite growth promotion (Olsson *et al* 1991; Bakhiet *et al* 1996), however, the role of IFN-γ in ruminant trypanosomiasis is not clear, but these results seem to indicate that it is protective.

IFN-γ is produced mainly by $CD8^+$, $CD4^+$ T cells and natural killer (NK) cells. Its target cells are mainly macrophages and monocytes but also B cells and T cells particularly $CD4^+$ T cells. The main biological effect of IFN-γ is to activate macrophages and it has been shown to have a beneficial effect of clearing intracellular parasites such as *Leishmania donovani, Trypanosoma cruzi* and *Plasmodium species* (Silva *et al* 1992; Sher and Coffman 1992). In contrast, during rat and mice infection with *T. brucei* it was identified as a trypanosome growth promoting factor (Ollson *et al* 1991; Bakhiet *et al* 1996). Based on studies in rodents infected with *T. brucei* a hypothesis was proposed that immunosuppression in trypanosomiasis is mediated by IFN-γ induced nitric oxide or prostaglandin from macrophages (Olsson *et al* 1992). However, Flynn *et al* (1991) demonstrated that prostaglandin was not involved in Boran cattle infected with *T. congolense* although macrophages from these animals were able to suppress naive mononuclear cell proliferation *in vitro*. Nitric oxide synthesis in *T. congolense* infected Boran cattle was down regulated from day 35 onwards while during the same period IL-10 was upregulated (Taylor, Lutje and Mertens 1996). Therefore, effects of IFN-γ in ruminant trypanosome infection might be different from those in rodents, and the mechanism of immunosuppression also seems to be different, probably involves IL-10 in ruminants.

In conclusion, these results demonstrate that ISMM sensitised sheep PBMCs *in vivo* for IFN-γ production and moderated proliferative responses to trypanosome antigens. It also induced down regulation of IL-12p40 production which inhibits IFN-γ production in mice. However, the pattern of IFN-γ modulation seems to be different in mice and sheep.

CHAPTER 8

CHAPTER 8
EFFECTS OF ISOMETAMIDIUM PROPHYLAXIS ON *BACILLE CALMETTE-GUERIN* (BCG) VACCINATION

8.1 INTRODUCTION

This experiment was conducted as a result of significant immunomodulation by ISMM in *T. congolense* infected sheep. Lymphocytosis and absolute B cells were significantly higher in the control group than in the prophylactic group. On the other hand absolute $CD4^+$ T cells were higher in the prophylactic group than in the control group. The working hypothesis was that ISMM immunomodulation was non-specific, therefore, it was expected to modulate cellular responses in *Bacille Calmette-Guerin* (BCG) vaccination, therefore, provide supporting evidence for the observed effects. BCG vaccine provided a non-trypanosome antigen that could be used to examine whether ISMM immunodulation was specific for trypanosome infections.

BCG contains a live attenuated *Mycobacterium tuberculosis bovis* used for immunising against the human form of tuberculosis caused by *Mycobacterium tuberculosis*. *M. t. bovis* is an intracellular ruminant pathogen whose immunological responses have been studied very extensively. It provides a good model for T cell mediated delayed-type hypersensitivity (DTH) immune responses to intracellular parasites.

M. t. bovis multiply in unsensitised macrophages and then spread to regional lymph nodes. The ability of macrophages to kill the bacteria is enhanced only after the development of cell mediated delayed type hypersensitivity reaction. After sensitisation (2 weeks onwards) lymphocytes release lymphokines that attract, activate and increase number of mononuclear cells around the infection site (Saito and Nakano 1996). Because *M. t. bovis* in BCG vaccine is known to induce cell mediated immune responses, it forms a good model for studying modulation of cellular immune

responses, hence the reason for using it in this experiment. Therefore, the objective of this experiment was to investigate effects of ISMM on PBMC responses to BCG vaccination, in order to determine whether immunomodulation by ISMM was specific to trypanosome antigens.

8.2 MATERIALS AND METHODS

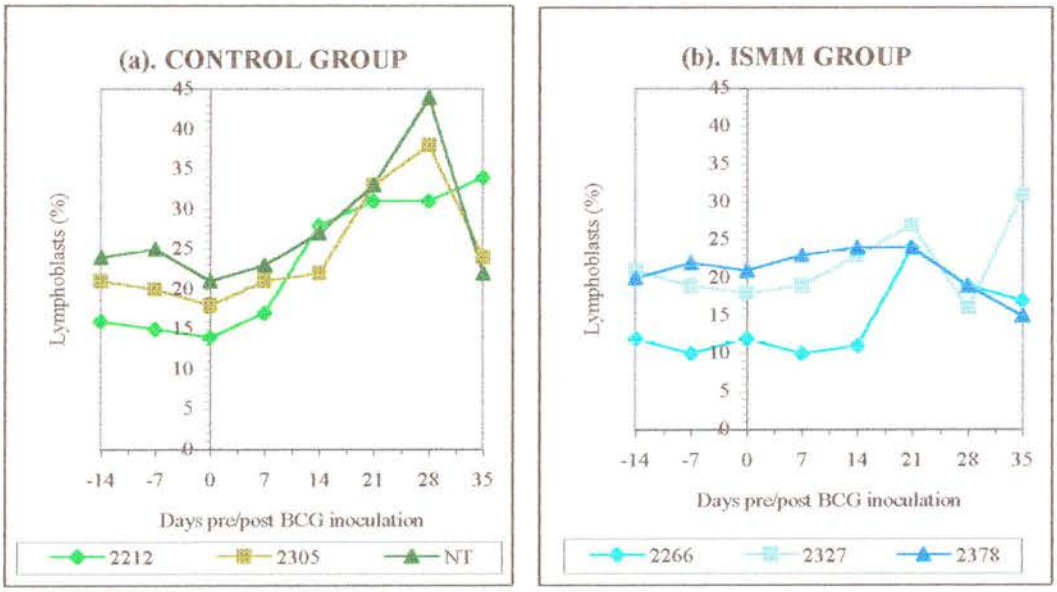
Peripheral blood mononuclear cell phenotypes (B cells, $CD4^+$, $CD5^+$, $CD8^+$ and $\gamma\delta^+$ T cells) as well as *in vivo* lymphocyte proliferation as indicated by an increase in lymphoblasts observed on flow cytometry were determined as described in Chapter 3. At the end of the experiment, 35 days after inoculation, Bovine tuberculin purified protein derivative (PPD) skin test was performed by injecting 0.2ml of PPD intradermally in the neck region. Skin thickness was then measured using a calliper daily for 3 days. PPD test is a standard diagnostic aid for detecting the presence of an active tuberculosis infection in ruminants.

8.3 RESULTS

8.3.1 *In vivo* PBMC proliferation (% Lymphoblasts) following BCG inoculation

The percentage of lymphoblasts in PBMC was estimated by Flow cytometry as described in Chapter 3 section 3.9.2. Dot plot results are presented in Appendix VI, Figures E1-E6. A significant increase ($P>0.01$) in lymphoblasts was recorded in the control group 7 to 35 days post BCG inoculation (Figure 8.1; Table E1-Appendix VI). Although sheep 2266 and 2327 in the ISMM group showed a slight increase 21 days after vaccination, the change above pre-vaccination levels was not statistically significant ($P>0.05$).

Figure 8.1 *In vivo* PBMC proliferation (% Lymphoblasts) following BCG inoculation

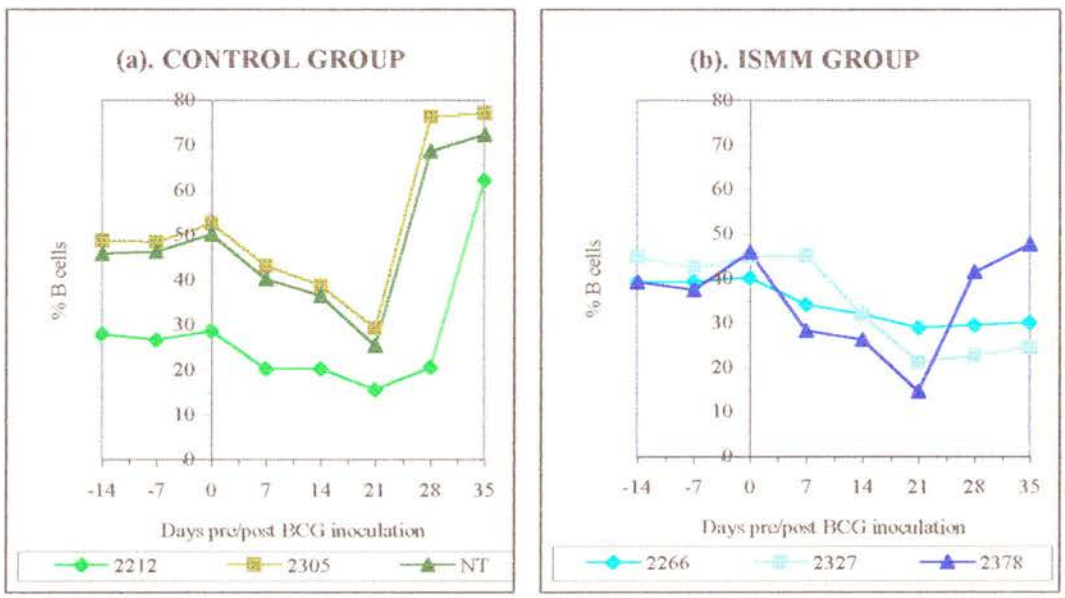


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.2 Percentage of B cells following BCG inoculation

Figure 8.2 and Table E2-Appendix VI, shows that B cells in the control group decreased significantly below pre-inoculation levels 7 to 21 days after inoculation, then increased above normal 28 days onwards ($P<0.05$). A significant decrease in the ISMM 7 to 21 days after inoculation ($P<0.01$) was followed by a return to normal thereafter. However, there was no significant difference in the percentage of B cells between the control and ISMM groups throughout the study period ($P>0.05$).

Figure 8.2 Percentage of B cells following BCG inoculation

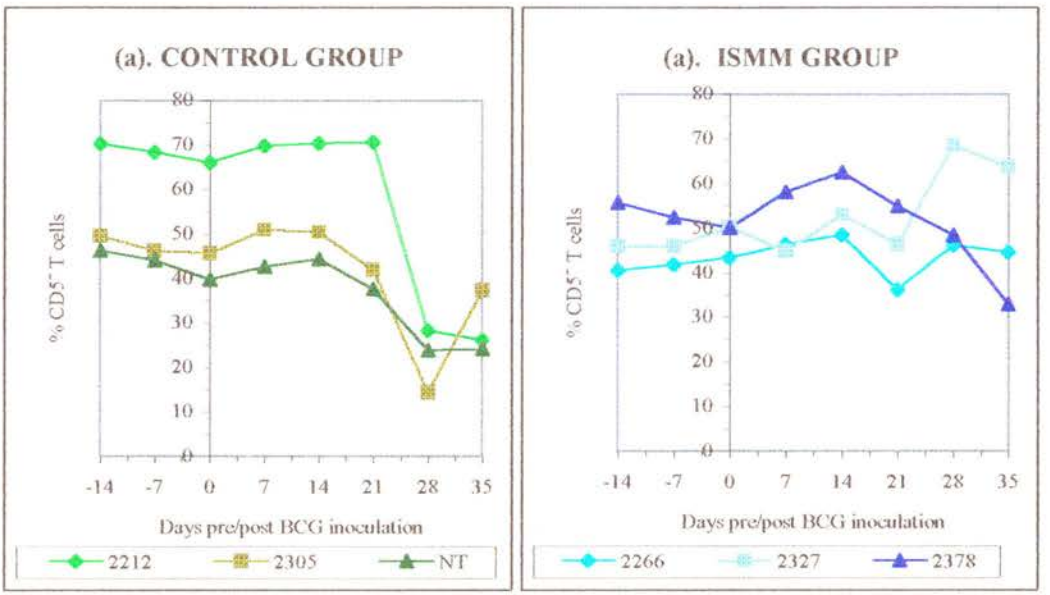


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.3 Percentage of $CD5^+$ T-cells following BCG inoculation

A significant ($P<0.01$) decrease in $CD5^+$ T cells was recorded in the control group 28 to 35 days after inoculation but not in the ISMM group (Figure 8.3; Table E3-Appendix VI,) leading to significantly ($P<0.01$) lower $CD5^+$ T cells in the control than in the ISMM group 28 to 35 days after vaccination.

Figure 8.3 Percentage of $CD5^+$ T-cells following BCG inoculation

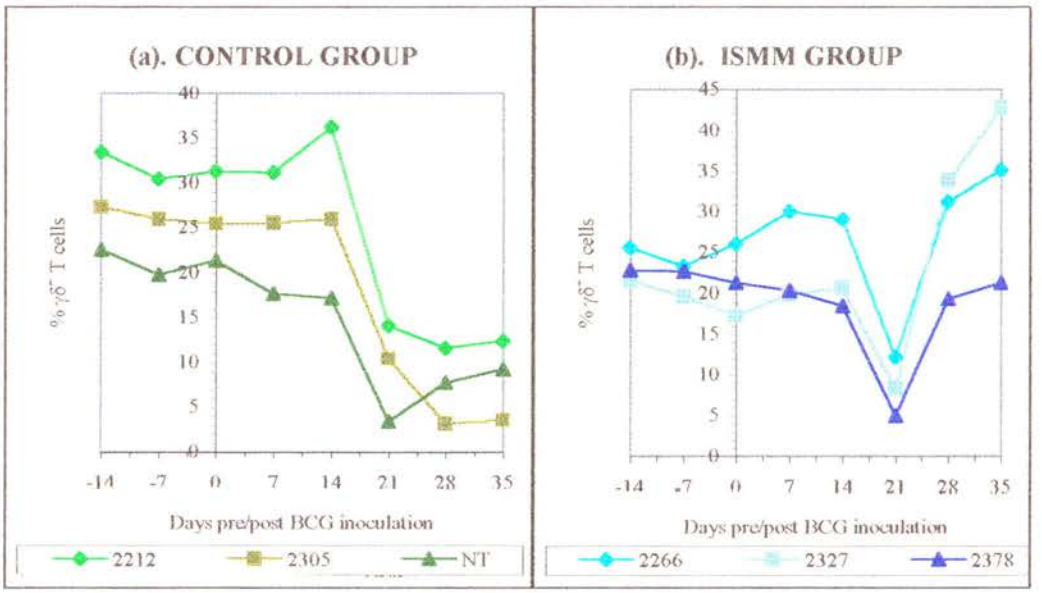


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.4 Percentage of $\gamma\delta^+$ T-cells following BCG inoculation

In the ISMM group, a transient decrease in the percentage of $\gamma\delta^+$ T cells was observed 21 after inoculation. While in the control group, a significant ($P<0.01$) decrease was observed 21 days onwards (Figure 8.4; Table E4-Appendix VI). Therefore, 28 days onwards the percentages of $\gamma\delta^+$ T cells in the two control group were significantly ($P<0.01$) lower than in the ISMM group.

Figure 8.4 Percentage of $\gamma\delta^+$ T-cells following BCG inoculation

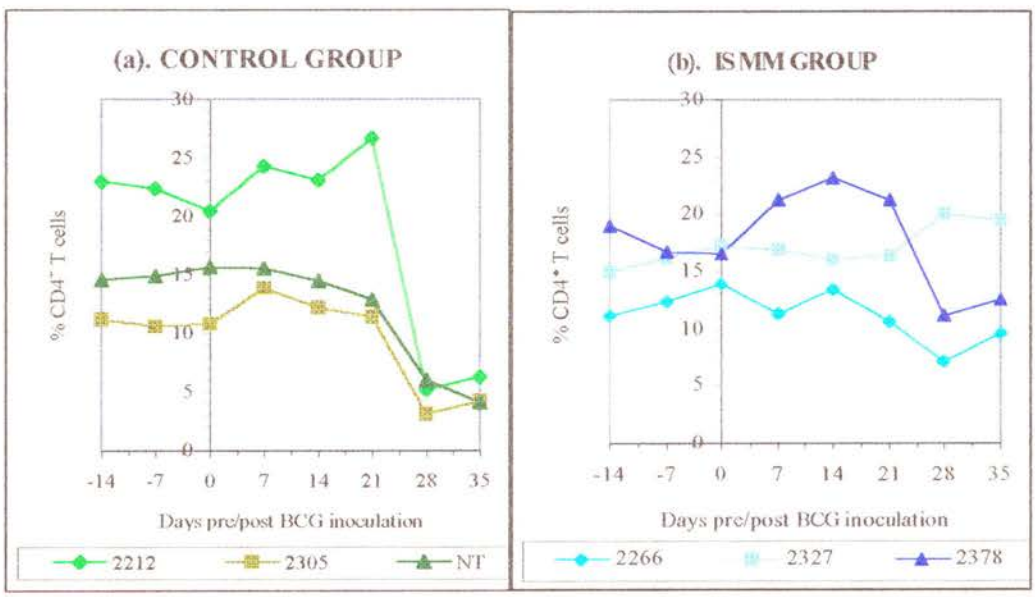


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.5 Percentage of $CD4^+$ T-cells following BCG inoculation

Variations in the percentage of $CD4^+$ T cells in the ISMM group were not significantly different from pre-inoculation levels. On the other hand, a significant ($P<0.01$) decrease was observed in the control group 28 to 35 days post inoculation. As a result of this, $CD4^+$ T cells in the control group were significantly ($P<0.01$) lower than those of the ISMM group 28 days onwards (Figure 8.5; Table E5-Appendix VI).

Figure 8.5 Percentage of $CD4^+$ T-cells following BCG inoculation

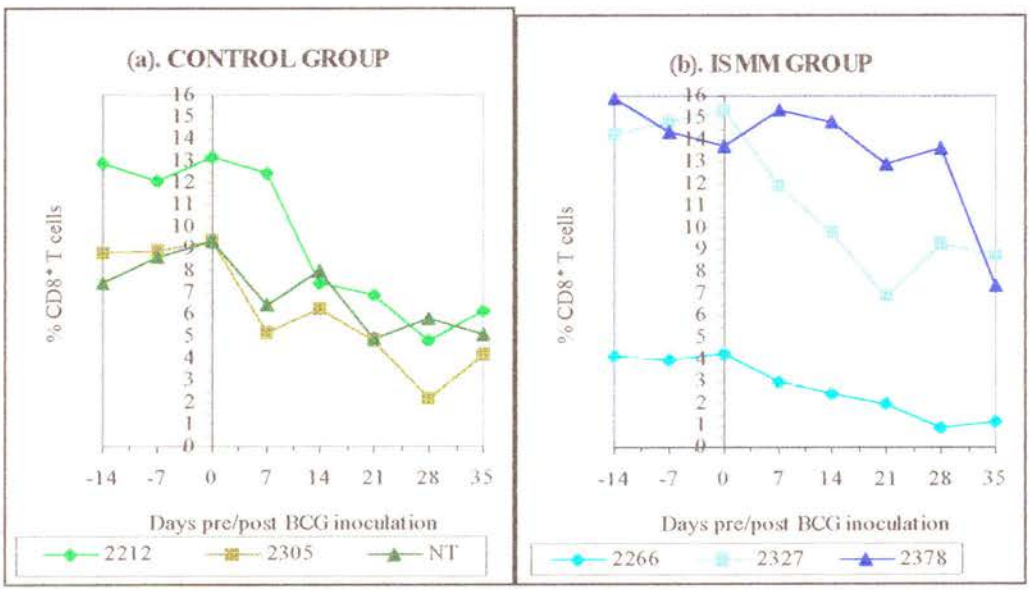


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.6 Percentage of $CD8^+$ T-cells following BCG inoculation

A decreased in the percentage of $CD8^+$ T cells after inoculation group was significant ($P<0.01$) in the control group. In the ISMM group, pre-inoculation percentages of $CD8^+$ T cells for sheep 2266 were much lower than the other two sheep (2327 and 2378) such that, the response after inoculation also varied greatly within the group. However, a non-significant decrease in all the sheep was observed. There was no difference in the pattern of response between the control and the ISMM groups (Figure 8.6; Table E6-Appendix VI).

Figure 8.6 Percentage of $CD8^+$ T-cells following BCG inoculation

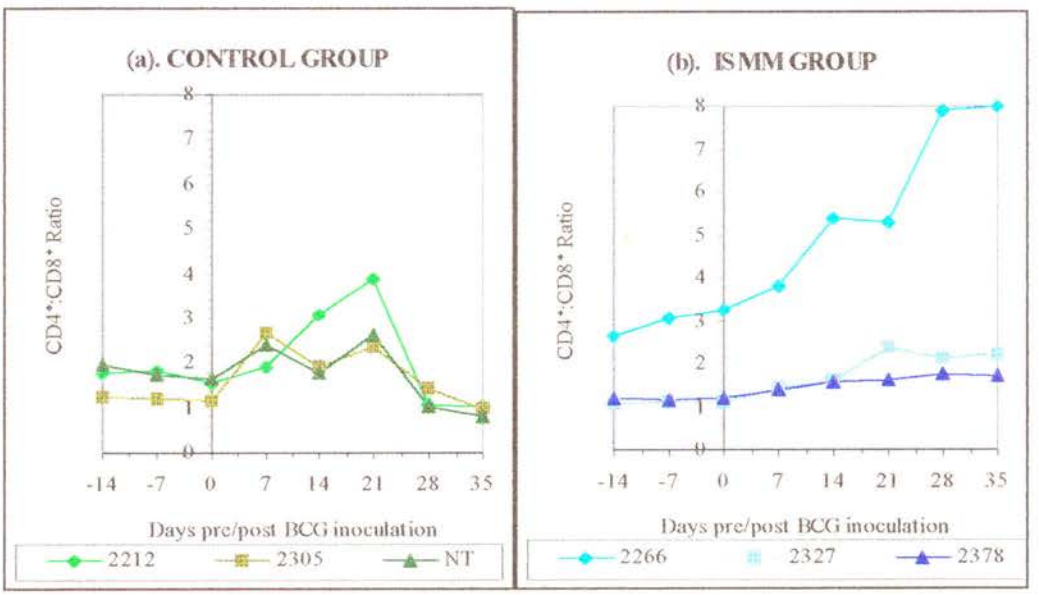


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.7 $CD4^+$:$CD8^+$ T-cell ratios following BCG inoculation

The ratio of $CD4^+$:$CD8^+$ T cells increased significantly ($P<0.01$) from 7 to 21 days after inoculation and returned to normal thereafter in the control group. In the ISMM a significant ($P<0.05$) increase from 7 until 35 days after inoculation was observed. Therefore, the ratio differed significantly ($P<0.01$) between the control and ISMM groups 28 to 35 days post vaccination. It had remained high in the ISMM group, but had returned to normal in the control (Figure 8.7; Table E7-Appendix VI).

Figure 8.7 $CD4^+$:$CD8^+$ T-cell ratios following BCG inoculation

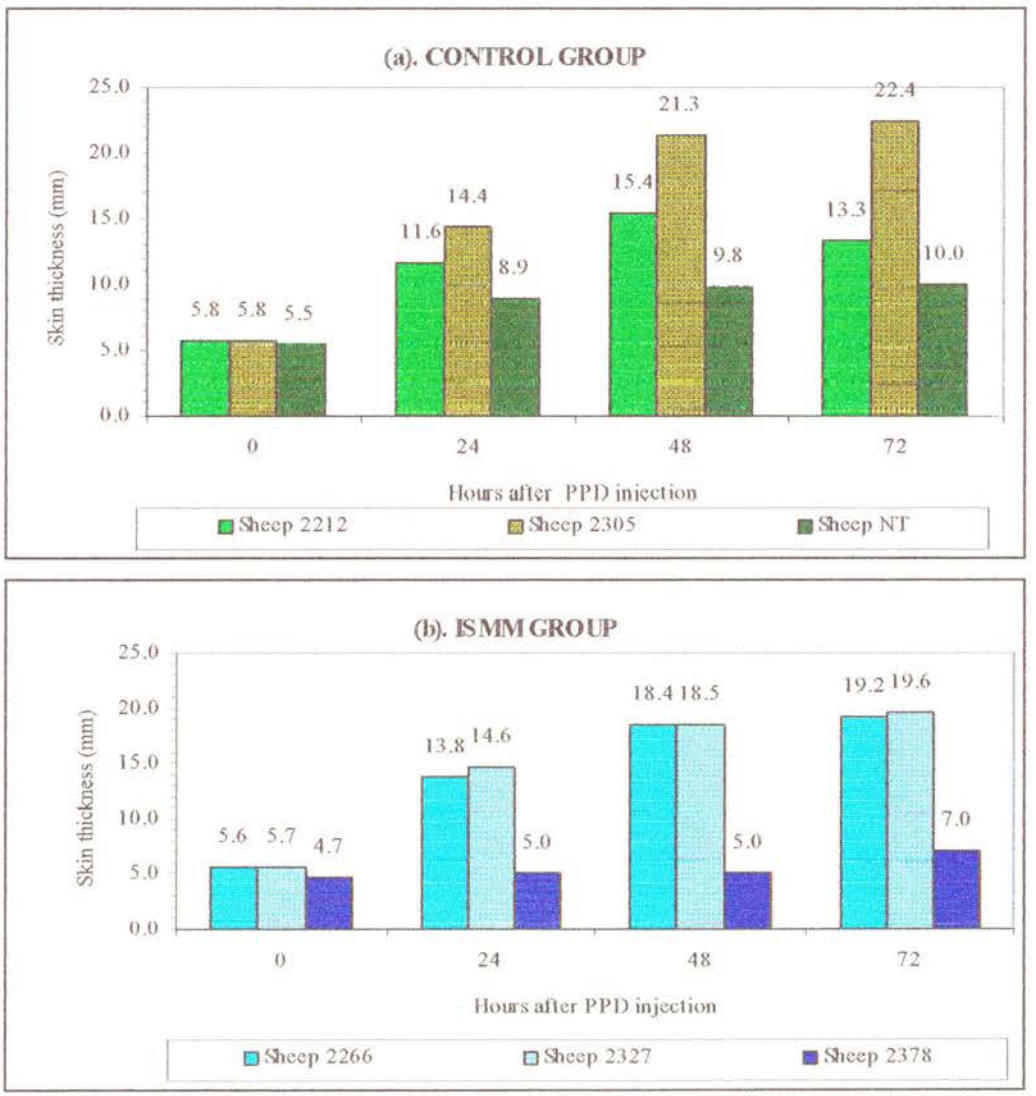


- ISMM group was treated 14 days before BCG inoculation
- All sheep in both groups were inoculated with BCG on day zero

8.3.8 Skin thickness (mm) after PPD injection 35 days post BCG inoculation

A positive reactor was considered to be any sheep that showed an increase in the skin thickness greater than 4mm, 72 hours after an intradermal PPD injection. Skin thickness in the control group had exceeded the critical value of 4mm by 72 hours after injection (Radostits *et al* 1994). Sheep 2378 in the ISMM group was a negative, while sheep 2266 and 2327 were positive reactors to PPD skin test. (Figure 8.8; Table E8-Appendix VI).

Figure 8.8 Skin thickness (mm) after PPD injection 35 days post BCG inoculation



8.4 DISCUSSION

At present, there are no reports in the literature on lymphocyte phenotype responses to *M. t. bovis* in ruminants, therefore these results are discussed by simply comparing pre- and post-inoculation values and relating then to responses observed in *T. congolense* infected sheep or cattle. Lymphoblasts in blood of the BCG control group started to increase 14 days after inoculation, peaked at 28 days and by 35 days post-inoculation, they were beginning to return to normal levels. On the other hand, in the ISMM group, no significant increase was observed following BCG inoculation. The same effect of ISMM on lymphocytosis was observed in trypanosome infected sheep. However, the lymphocytosis the first 3 weeks after inoculation was largely due to an increase in T cells in BCG vaccinated sheep while in trypanosome infected sheep, a slight T cell increase for 7 days was followed by a large increase in B cells 14 days onwards. These results imply that ISMM moderated both T and B cell polyclonal proliferation. In turn, this could inhibit the development of clonal anergy which normally results from polyclonal expansion of both effector and memory cells. Excessive clonal expansion has been shown to lead to clonal exhaustion or anergy in trypanosome infections, a situation in which immune cells become unresponsive (Urquhart *et al* 1973 review).

The percentage of B cells in the control group decreased while lymphocytosis was developing 7 to 21 days after inoculation. Therefore, absolute B cells remained constant during this period in the control group, but 28 days onwards, started to increase. On the other hand in the ISMM group, 7 to 21 days after inoculation, there was little or no lymphocytosis, and there was a decreased percentage of B cells, resulting in a constant absolute B cell count and no observed changes thereafter. B cell responses seemed to be different in BCG vaccinated and trypanosome infected sheep. In trypanosome infected control sheep, absolute B cells increased for 21 days and began to decrease 28 to 35 days post-infection, while in BCG sheep there was no change for 21 days but started to increase 28 days onwards. However, ISMM

inhibited polyclonal B cell proliferation in BCG vaccinated and trypanosome infected sheep.

Seven to twenty one days after inoculation there was lymphocytosis, and a constant percentage of $CD5^+$ T cells in the control group. This means, during this period, there was an increase in absolute $CD5^+$ T cells in the control group. But by 28 days onwards, $CD5^+$ T cells were returning to normal. On the other hand in the ISMM group, 7 to 21 days after inoculation, there was little or no lymphocytosis, with a constant percentage of $CD5^+$ T cells. This implies that during this period there was little or no increase in absolute $CD5^+$ T cells. In trypanosome infected sheep a similar pattern of response was observed, in the control and the prophylactic groups. In BCG vaccinated as well as trypanosome infected sheep, ISMM inhibited changes in $CD5^+$ T cell responses.

The percentage of $CD8^+$ T cells decreased in the presence (control group) and absence (ISMM group) of lymphocytosis. This means the absolute $CD8^+$ T cells decreased in both groups throughout the period. In trypanosome infected sheep, there was also no difference in $CD8^+$ T cell responses in control and prophylactic groups. Therefore, ISMM did not seem to affect $CD8^+$ T cell responses.

Changes in the percentage and absolute $CD4^+$ T cells followed the same pattern as that of $CD5^+$ T cells in both groups. That means in the control group, 7 to 21 days after inoculation there was an increase in absolute $CD4^+$ T cells followed by a return to normal thereafter, while in the ISMM group, there was little or no change in absolute $CD4^+$ T cells. In trypanosome control sheep, a non-significant increase in absolute $CD4^+$ T cells 7 to 14 days post infection was followed by a significant drop below normal levels 21 days later, while no significant changes were observed in the prophylactic group. This is an indication that ISMM affected $CD4^+$ T cell responses by inhibiting polyclonal probably in favour of monoclonal expansion and by inhibiting activation induced cell death.

The ratio of $CD4^+$:$CD8^+$ T cells in the control group increased 7 to 21 days after inoculation but returned to normal thereafter. In the ISMM group, an increase in $CD4^+$:$CD8^+$ T cells was observed throughout the study period. In trypanosome control sheep an increase in the ratio was observed 7 to 14 days after infection, but decreased significantly below normal levels 21 days later, when an increase was being observed in the prophylactic group. The end result was that there were unusualy more $CD4^+$ T cells than $CD8^+$ T cells in the ISMM group.

In the control group, the percentage of $\gamma\delta^+$ T cells remained constant 7 to 21 days after inoculation when there was lymphocytosis, but decreased 28 days onwards when lymphocytosis had subsided. Therefore, absolute $\gamma\delta^+$ T cells increased 7 to 21 after vaccination and returned to normal thereafter. In the ISMM group, both the percentage and absolute $\gamma\delta^+$ T cells showed a transient decrease 21 days after inoculation. In trypanosome controls, a non-significant increase in $\gamma\delta^+$ T cell was recorded 7 to 14 days after infection and was followed by a decrease below normal levels 21 days later, while in the ISMM group a decrease 7 to 14 days after infection was observed. Therefore, ISMM appeared to have inhibited an increase in $\gamma\delta^+$ T cells that occurred between 7 and 14 days in control groups of both trypanosome infected and BCG vaccinated sheep.

The skin thickness had increased by more than 4mm in all sheep except sheep 2378 from the ISMM group which did not react. The PPD skin test may fail to detect cases of minimal sensitivity as may occur in early or late stages of *Mycobacterium bovis* infection. Non-reactors may also occur due to desensitisation caused by absorption of tuberculin and other foreign proteins (Radostis *et al* 1994). Desensitisation may last long after subcutaneous than after an intradermal injection. However, sheep 2378 seemed not to have been properly sensitised (might have been underdosed) by the BCG vaccine because it was the only one in the group with odd responses for most parameters tested even before the PPD skin test was performed. However, the reaction to the PPD skin test was not significantly different between the groups, implying that despite modulation of certain aspects of the immune system, the actual

reaction to the PPD skin test was not significantly different between the groups, implying that despite modulation of certain aspects of the immune system, the actual response to challenge was not affected or that data was not sufficient to detect an significant difference.

In conclusion, ISMM modulated cellular responses to BCG vaccination in sheep by favouring an increase in $CD4^+$ T cells with respect to $CD8^+$ T cells, moderating polyclonal B cell proliferation and lymphocytosis in a way similar to effects in *T. congolense* infected sheep. This provides further evidence that the difference in immunological responses between the *T. congolense* control and prophylactic group is not a result of direct destruction of trypanosomes since there is no evidence that ISMM is bactericidal at prophylactic levels.

CHAPTER 9

CHAPTER 9
GENERAL DISCUSSION

Because of the major economic importance of animal trypanosomiasis, the majority of control strategies aimed primarily at the protection of these animals use ISMM as a major component of their programmes. Although there are many different ways of combating animal trypanosomiasis, each of the control methods available has serious limitations. The high cost involved in conducting tsetse eradication programmes is often beyond the reach of individual farmers or countries. Furthermore, tsetse fly clearance is not a reliable control measure if continued surveillance cannot be guaranteed. Nevertheless, even tsetse fly eradication will not necessarily result in the eradication of trypanosomiasis since *T. vivax* and *T. evansi* can cause infection without cyclical transmission and can be spread mechanically by biting flies. Furthermore, tsetse fly eradication by aerial or ground sprays of massive amounts of chemicals may have adverse environmental effects (Ford 1971). Chemicals sprays despite being effective kill insects and other animals indiscriminately.

Conserving biological diversity has emerged as a priority shared by conservation and many development projects including tsetse and trypanosomiasis control projects. Tsetse distribution in many countries is closely associated with wildlife habitats and adjacent areas. Therefore, emphasis on tseste eradication and livestock production indirectly threatens the survival of wildlife. It can now be urged that tsetse and trypanosomiasis although generally considered to be a problem to man and his livestock, has prevented people from claiming more and more land from wildlife habitats. National parks and wildlife reserves are at the forefront of conserving biological diversity and its probably time livestock development became integrated with wildlife management in order to avoid conflict of interests. For instance, people living in tsetse infested areas could be encouraged to keep and manage wildlife ranches instead of livestock as a source of income through tourism and sales of game meat and trophies. As a matter of fact, these days some wild animals are more valuable than livestock but can survive in harmony with tsetse and trypanosomiasis at

very minimal cost. Examples of existing integrated conservation projects which are increasingly involving the local people in wildlife management include: the Lupande/ADMADE and Luangwa Integrated Rural Development Project (Zambia); East Usambara (Tanzania), Amboseri National Park (Kenya) and Volcanoes National Park in Rwanda (Wells, Brandon and Hannah 1992). If such projects could include among their aims, the phasing out of livestock rearing in the vicinity of wildlife habitats in favour of game animals, the benefits could be phenomenal.

In the absence of a lasting solution to conserving biodiversity in relation to wildlife and livestock development, tsetse fly control strategies using targets or traps continue to be used. Tsetse flies can detect odours by means of receptors on their antennae. The knowledge of insect pheromones was used to detect the chemical component of cattle odour which would attract tsetse flies and led to the discovery of 1-octen-3-ol. It proved highly attractive to *Glossina m. morsitans* and *G. pallidipes* (Allosopp, Hall and Jones 1985). Traps baited with acetone and 1-octen-3-ol are used in many areas of Africa. Targets, which are also baited with acetone and 1-octen-3-ol consist of a piece of black or blue cloth (colours that also attract tsetse flies) and sprayed with an insecticide such as deltamethrin. Attracted flies land on the target and receive a lethal dose of the insecticide. Targets and traps are less indiscriminate since they kill only flies that are attracted to them. However, traps and targets, despite being cheaper than aerial or ground sprays only manage to reduce the risk of infection and not eradicate it.

In regions with low or moderate tsetse fly density, where eradication of the fly is not possible with present methods, genetic improvement of trypanotolerant animals could be attempted. Such programmes could involve selection of trypanotolerant animals under natural challenge or selection of marker traits such as aspects of immune response associated with resistance to trypanosomes. A *T. congolense* cysteine kinase (congopain) elicits a high IgG response in trypanotolerant but not in trypanosusceptible cattle during primary infection. It is suggested that the difference might reflect a dysfunction in the isotype switch mechanism in infected susceptible

Zebu cattle. Therefore, regardless of the mechanism involved the antibody response to congopain in primary infection appears to be a marker of trypanotolerance (Authie 1994). Congopain is a cystein protease produced by trypanosomes and other micro-organisms which has been shown to degrade host proteins such as immunoglobulins and complement factors. However, it can also be immunogenic. Studies of immune responses in antelopes such as buffaloes and elands could also assist in identifying resistance markers. Many wild animals co-exist with tsetse transmitted trypanosomes and African buffalo and eland serum contain trypanocidal protein that is able to kill blood stream forms of *T. congolense, T. vivax, T. evansi, T. brucei, T. gambiense* and *T. rhodesiense* within 4 hours *in vitro* (Reduth *et al* 1994). However, this trypanocidal material is not lipoprotein, IgG or IgM. Trypanotolerance is probably multifactorial and appears to involve both immunological and non-immunological mechanisms. Whether trypanotolerance is determined by innate mechanisms or by acquired immunity to trypanosomes is not yet known. Nevertheless, in areas of high tsetse fly challenge even trypanotolerant animals will not survive unless they are treated prophylactically against trypanosomes.

Thus, in the absence of a suitable vaccine or any other more effective method, ISMM prophylaxis forms the most important part of most trypanosomiasis control measures. However, the use of ISMM in trypanosomiasis control programmes is not without problems. The manufacturers recommend the use of ISMM at a dosing interval of 3 months. At this frequency of use, ISMM is generally considered to be cost effective. However, it is very common for a considerable number of animals to become infected by the end of one month even after careful drug administration (Fairclough 1963; Pinder 1984; Eisler *et al* 1994). Therefore, a dosing interval of less than 3 months generally ceases to be cost effective and increases treatment costs or animal losses.

In order to correct the problem of prophylactic drug failure, a substantial understanding of protective mechanisms involved is vital because the conventional concentration (or dose)-response relationship that applies to most drugs does not apply to ISMM. Most drugs that have a direct parasite killing capacity have a

minimum effective concentration (MEC_{100}) that would kill all parasites *in vitro* and *in vivo*. The MEC_{100} for ISMM against different strains of trypanosomes *in vitro* has proved difficult to define because of varying sensitivities to the drug. *In vitro* studies with ISMM demonstrated that the MEC_{100} for *T. evansi* and *T. equiperdum* after 24 hours of drug exposure was 1 000 - 4 000 ng/ml (Zhang, Giroud and Baltz 1991) and 1 - 300 ng/ml after 96 hours of exposure (Brun and Lun 1992). Also culturing *T. congolense* and *T. vivax*, *in vitro* in the presence of 5 ng/ml ISMM for 10 minutes (Sutherland, Mounsey and Holmes1991), 10 ng/ml for 24 hours (Kaminsky, Chuma and Wasike 1994) and 10 ng/ml for 48 hours (Gray and Peregrine 1993) could not completely eliminate parasites or prevent infection in mice. Results from this study has also shown that ISMM could not kill all trypanosomes *in vitro* at 100 ng/ml for 48 hours. These studies demonstrate that the MEC_{100} for the common *T. congolense* isolates in greater than 10ng/ml. It is known that trypanosome sensitivity assays carried out *in vitro* are "closed systems" where all metabolic and breakdown products are not eliminated and the drug concentration is not subjected to elimination processes that exist *in vivo*. Therefore, survival of parasites for more than 24 hours in such harsh *in vitro* environments in the presence of ISMM is an indication of an ineffective killing capacity of ISMM drug residues.

The plasma profile of ISMM in treated sheep showed that the concentration was below 3 ng/ml 21 days after administration, such that by the time of infection four and a half months later, drug levels should have been undetectable. Yet the establishment of *T. congolense* did not occur. These results demonstrated that ISMM is more effective *in vivo* than it is *in vitro*, probably as a result of potentiation of the immune system. Several other experiments have demonstrated *in vivo* protection due to ISMM at very low or undetectable plasma or serum levels (Eisler *et al* 1994; Geerts *et al* 1997).

The mechanism of protection when ISMM plasma levels are low or undetectable is still not well known. Nevertheless, because no trypanolytic antibodies exist in prophylactically treated animals, the protection has been attributed to drug residues

(Whitelaw *et al* 1986; Geerts *et al* 1997). However, the fact that trypanolytic antibodies may not be detected in serum of animals under prophylactic cover does not rule out the involvement of certain other aspects of the immune system. There are suggestions in the literature that the immune system may be involved in the long term protective effects of ISMM, the only problem has been the inability to prove their existence. Hill (1965) demonstrated that activity of ISMM against *T. congolense in vitro* was less than that observed *in vivo*. These differences could have been attributed to the possibility of potentiation of the immune system *in vivo*.

In this study trypanosome specific IgG antibodies were almost twice as high in the prophylactically treated sheep compared to the control group. Nevertheless, they were almost absent in the group of sheep treated 14 days after infection. These results demonstrate that ISMM prophylaxis results in an up-regulation of IgG antibodies which are involved in opsonisation and phagocytosis. On the other hand treatment of an established infection with ISMM may lead to suppression of IgG antibodies. In primary infections in mammals, initial IgM antibody synthesis is usually replaced by the synthesis of IgG antibodies (Uhr 1964). However, in trypanosome infections, IgM antibodies remain characteristically high through out the infection (Luckins 1976), but they maybe augmented by IgG antibodies (Nantulya, Musoke and Moloo 1986). Variable surface glycoprotein specific activity is found in both IgG and IgM fractions. IgM fractions are more efficient at neutralisation, agglutination and lysis *in vitro* compared to IgG antibodies.

However, it is more likely that clearance of antibody coated trypanosomes *in vivo* occurs primarily by phagocytosis and there is considerable evidence in support of this view. *In vitro* studies have shown that *T. brucei* and *T. gambiense* are ingested by macrophages in the presence of immune serum (Lumsden and Herbert 1967). *In vivo* evaluation of the role of opsonisation and macrophage uptake of radiolabeled trypanosomes showed that whereas labelled parasites remained in circulation of normal mice, they were rapidly eliminated from blood of immune animals. In the immunised mice the liver was found to be the principal site of uptake, removing over

50% of trypanosomes within 15 minutes of an intravenous injection of labelled parasites (Holmes *et al* 1979; MacAskill *et al* 1980). The possibility that immune lysis was a pre-requisite to phagocytosis of trypanosomes was investigated *in vivo* by measuring trypanosome clearance ability of passively immunised C5-deficient and C3-depleted mice (MacAskill *et al* 1980). Results of this experiment showed that C5-deficiency which is necessary in complement mediated lysis did not affect immune clearance, while reduction in C3 which is essential for full opsonic activity prevented immune clearance in passively immunised mice.

IgG antibody production was enhanced in the prophylactic group and suppressed in those treated with ISMM 14 days after infection. The difference is likely to be due to the status of the host regulatory cytokines at the time of infection. Numerous cytokines are produced by a variety of cells after infection, but IFN-γ production was investigated in this study because of its ability to down-regulate proliferation of mononuclear cells from *T. brucei* infected rodents (Olsson *et al* 1991; Bakheit *et al* 1996). IFN-γ is produced by many cell types including $CD8^+$, $CD4^+$, $\gamma\delta^+$ T cells and natural killer cells. Biological effects of IFN-γ include: increased macrophage activity and cytokine production such as TNF; induction of IL-2 and IL-2R expression by T cells; decreased B cell proliferation and the promotion of IgG2a antibody isotype production (Tizard 1992).

IFN-γ production in sheep was investigated as a possible target for ISMM induction leading to modulation of cellular and antibody responses. PBMC collected 14 to 21 days after ISMM administration from the prophylactic non-infected sheep produced significant amounts of IFN-γ when cultured *in vitro* in the presence of live trypanosomes. ConA cultures produced IFNγ in a manner that was not related to the time after drug administration. These results indicate that ISMM activated naive PBMCs *in vivo* for IFN-γ production *in vitro* when cultured with trypanosomes. The heightened responsiveness was maintained in one sheep until 21 days after infection when it dropped to normal levels, despite the fact that infection was not established. In the literature there are no reports of IFN-γ production by PBMCs from either naive

or trypanosome infected animals when cultured with trypanosome antigens. The results of Lutje *et al* (1995) showed that high levels of IFN-γ were detected from *in vitro* trypanosome antigen culture supernatants of lymph node cells in Boran cattle 11 days after infection with *T. congolense*, but not from trypanosome-PBMC culture supernatants. Similar results were obtained by Taylor, Lutje and Mertens (1996) who were able to detect IFN-γ from lymph node cells 11 and 31 days after infection. Thus, sensitisation of lymphocytes in prophylactically treated sheep for IFN-γ production appears to be one mechanism resulting in the modulation of cellular and antibody responses after challenge with trypanosome antigens.

Naive lymphocytes are pluripotent, therefore they can differentiate along different pathways to become effector cells depending on the additional signal received during activation. These additional signals are induced by pathogens in the effector cells of the innate immune system (Romagnani 1992). Different effector cells produce different sets of cytokines when stimulated by antigens. Type 1 cytokines which includes IFN-γ and IL-12 induce the differentiation of T cells into Th_1 effector cells and IgG_2 antibody production favouring cell mediated repsonses. Type 2 cytokines (IL-4 and IL-10) induce the Th_2 differentiation pathway and IgG_1 and IgE antibody production in favour of humoral immune responses (Sender and Paul 1994). However, Th_0 effector cells which produce both IFN-γ and IL-4 also exist. Therefore, the innate non-clonal system controls the initiation of the clonal adaptive immune response by regulating the expression of co-stimulatory activity on antigen presenting cells. A signal received through an antigen receptor is not sufficient on its own for the activation of naive lymphocytes. A second, so called, co-stimulatory signal is required for lymphocyte stimulation to occur. Co-stimulators are members of the immunoglobulin superfamily especially B7.1 and B7.2 molecules (June *et al* 1994). In the absence of the second signal no response occurs and T cells become anergic while in the presence of a signal through the T cell receptor and the co-stimulator molecule, the T cell is activated to subsequently express other interactive surface molecules such as TNF, Fas ligand and IL-2R, as well as the secretion of cytokines (June *et al* 1994). T cells and cell lines derived from *T. brucei* infected mice

appeared exclusively to represent Th_1 cells specific for the variant surface glycoprotein determinants. In contrast T cells and cell lines from immunised mice represented a more diverse T cell responses in which Th_0, Th_1 and Th_2 subsets were generated (Mansfield 1994). Therefore, differences in cytokine profile present at the time of infection might explain why IgG antibody responses in the prophylactic and treated groups were different although the exact mechanism is unknown.

When live *T. congolense* were cultured with splenic cells from either ISMM treated or un-treated mice for 24 hours, there was no induction of IFN-γ production. However, LPS induced IFN-γ production only in splenic cells from un-treated mice, implying that ISMM suppressed LPS induction of IFN-γ production. The role of IFN-γ in down-regulating mononuclear cell proliferation in rodents was demonstrated by culturing cells in the presence or absence of anti-IFN-γ antibodies. Results indicated that cells cultured in the presence of antibodies proliferated while those without antibodies to IFN-γ did not proliferate (Olsson *et al* 1991). Down-regulation was effected by a feedback mechanism resulting from IFN-γ activated macrophages mediated by prostaglandins (Schleifer and Manfield 1993) and nitric oxide (Schleifer and Manfield 1993; Sternberg and MacGuigan 1992). However, the pattern of IFN-γ production obtained in mice was different from that obtained in sheep. In sheep the response increased 14 to 21 days after treatment while in mice, the response was high in naive and was not there in prophylactically treated mice. Nevertheless, the role of IFN-γ in ruminant trypanosomiasis is not clear, but the association of IFN-γ production with ISMM protection in this study may be suggestive of a protective role since so far a direct link with the proposed mechanism leading to immunosuppresion in rodents seems to be well regulated in ruminants (Darji *et al* 1991; Taylor, Lutje and Mertens 1996).

Parasites usually interact directly with antigen presenting cells to induce the production of IL-12 which is a potent inducer of IFN-γ production on T cells. Therefore, IL-12 production was investigated because of its important role in regulating IFN-γ production and the subsequent development of the acquired immune

responses. Assessment of IL-12p70 in the same mice splenic cell cultures that were assayed for IFN-γ showed that none of the trypanosome or LPS cultures were positive for IL-12p70 indicting that it was either not produced or levels were too low to be detected by the assay. On the other hand IL-12p40 levels were high in both LPS and trypanosome cultures of non-treated as well as those obtained 7, and 14 day after ISMM administration but dropped significantly in cells collected 21 days after ISMM administration. The absence or low levels of IL-12p70 and presence of high levels of IL-12p40 could have resulted in a down-regulation of IFNγ production in ISMM treated mice splenic cell cultures. More studies on IFN-γ and IL-12 production are required since these were only a preliminary experiment aimed at elucidating the mechanism of immunomodulation resulting from ISMM administration.

Regulation of IL-12 production is by negative and positive feedback mechanisms involving IFN-γ as the most potent up-regulator (Kubin, Chow and Trinchieri 1994) and IL-10 as the most important down regulator (D'Andrea *et al* 1993). Because of the toxic and in some cases lethal effects of IL-12 overproduction, it is necessary that effective mechanisms should exist *in vivo* to limit the production or the ability of T cells and natural killer cells to respond to it. Currently, IL-10 produced mainly by activated macrophages, is the most effective down-regulator of the biological effects of IL-12 (D'Andrea *et al* 1993). Biological effects of IL-12 include: induction of several cytokine production especially IFN-γ by T and natural killer cell; promotion of haematopoiesis; enhancement of cell-mediated cytotoxicity (Mansfield 1995). It has been demonstrated that if IL-12p70 and IFN-γ are present during early T cell expansion in response to antigen, Th_1 cell generation is favoured and the generation of Th_2 cells inhibited. Mansfield *et al* (1993), reviewed the existence of both Th_1 and Th_2 responses in trypanosome infections. However, it is not yet clear which one of these responses may be associated with pathology or resistance to different species of trypanosome infections. Results of this study suggest that ISMM prophylaxis favours an early presence of Th_1 cytokines resulting in the enhancement of immune responses.

An experiment set up to determine the effect of suppressing cell-mediated immune responses on the efficacy of ISMM in mice showed that the prepatent period and survival time in mice that had received hydrocortisone (a cell-mediated immune response suppressor) and ISMM was the same as that observed in the control group. On the other hand, even though all mice developed parasitaemia and died, those that received ISMM only, had a long prepatent period and survived longer. The major immunosuppressive effects of hydrocortisone include: a decrease in concentration of various complement components; reduced proliferative responses to mitogens and antigens *in vitro*; decreased cytokine production (e.g. IL-2, IL-1) and decreased sensitivity of macrophages to cytokines, resulting in decreased phagocytosis. This implies that suppressing cellular responses using hydrocortisone reduced the ability of ISMM to control the establishment of the infection. It is known that the dose of ISMM required to prevent or treat *T. congolense* infection effectively in mice is about ten times that for ruminants (Sones *et al* 1988). However, in this study the same dose of 1mg/kg body weight was used in sheep and mice in order to make comparisons relevant.

Three groups of sheep were used to investigate further the effects of ISMM on cellular immune responses. A control group, ISMM prophylactic group, and another that had to be treated with ISMM 14 days after infection, were infected with *T. congolense*. Results showed that none of the sheep in the prophylactic group became parasitaemic throughout the study period, while all sheep from the control and treated groups became parasitaemic 7 days after infection. The control group remained parasitaemic, while after treatment parasites were cleared from the ISMM-treated group. The magnitude of fluctuating fever was highest in the control group while in the treated group it subsided after treatment. None of the sheep in the prophylactic group became febrile. Comparing N'dama and Boran cattle undergoing a primary infection with *T. congolense*, Williams *et al* (1991) found that parasitaemia was lower and usually intermittent in the trypanotolerant N'dama than in the susceptible Boran cattle. The results of this study demonstrate that ISMM prophylaxis was capable of preventing the establishment of a *T. congolense* infection four and a half months after

treatment and that a similar dose was also capable of clearing an established infection. Complete protection was also afforded by ISMM for four months (Peregrine *et al* 1988), for one to six months (Eisler *et al* 1994) in Boran cattle and for 20 months using a sustained release device (Geerts *et al* 1997). Thus, ISMM does indeed protect animals from *T. congolense* infection even at very low or undetectable plasma concentrations although the duration of protection varies widely.

Anaemia, assessed by measuring the PCV, showed a significant decrease from 14 days after infection till end of experiment 35 days later in the control group. The PCV in the treated group returned to normal about one week after treatment and no change was observed in the prophylactic group. This showed that ISMM prevented the development of pathological lesions and enabled animals to recover quickly when treated. Anaemia is one of the major pathological lesions associated with animal trypanosomiasis. The onset and degree of anaemia are closely associated with the development and level of parasitaemia. Trypanotolerant N'dama cattle, unlike susceptible Zebu, have the ability to control the parasitaemia and hence the loss of red cells. Dargie *et al* (1979) observed that the PCV decreased to a steady state of 22% in Zebu and 27% in N'dama from a mean of 35%. However, parasitaemia in N'dama cattle fell to undetectable levels 5 weeks after infection while Zebu cattle entered a chronic phase of infection. Therefore, the management of the disease in prophylactically treated animals is similar but more effective than that observed in trypanotolerant N'dama cattle. Increases in absolute monocyte count were observed in the control and treated groups 14 to 21 days after infection. There were no significant monocyte changes in the prophylactic group. Absolute neutrophil and eosinophil counts did not show any significant changes in all groups. Therefore ISMM prophylaxis prevented both the establishment of the infection and development of pathological lesions but allowed the initiation and development of immunological responses.

Total white blood cell (WBC) counts increased significantly in the trypanosome control group 14 days post infection till end of experiment 35 days post infection. The

pattern of increase in WBC counts was similar to that of absolute lymphocyte counts because lymphocytosis was the major contributing factor to increased WBC count. Lymphocytosis was observed during the first 21 days after BCG vaccination in the control group which was largely due to an increase in $CD5^+$ T cells followed by an increase in B cells 28 days onwards, while in trypanosome infected sheep a slight $CD5^+$ T cell increase for 7 days was followed by a large increase in B cells 14 days onwards. On the other hand, no significant lymphocytosis was observed following BCG inoculation in the ISMM group. Similarly, there was no lymphocytosis in the trypanosome prophylactic group. Therefore, ISMM prevented the severe lymphocytosis in trypanosome infected and BCG vaccinated sheep that results from $CD5^+$ T cell and polyclonal B cell proliferation. The beneficial effects of this type of modulation is the inhibition of the development of clonal anergy which normally results from polyclonal expansion of both effector and memory cells. Excessive clonal expansion has been shown to lead to clonal exhaustion or anergy in trypanosome infections, a situation in which immune cells become unresponsive (Urquhart *et al* 1973 review). These results indicate that the effect of ISMM on lymphocytosis is not necessarily due to direct destruction of trypanosomes, since it is unlikely that ISMM has bactericidal effects against *M. tuberculosis bovis*. Total leucocyte and lymphocyte numbers returned to normal in the trypanosome experiment 2 weeks after treatment. Lymphocytosis and leucocytosis were also demonstrated, by Mwangi (1991), in sheep infected with *T. congolense* 25 days after infection onwards and in cattle infected with *T. congolense* (Williams *et al* 1991; Valli and Forsberg 1979).

In general, absolute numbers and the percentage of $CD8^+$ T cells decreased after trypanosome infection or BCG vaccination in control or prophylactic groups. An increase in $CD8^+$ T cells was observed only in trypanosome infected and treated sheep soon after treatment. Therefore, ISMM prophylaxis had no effect on $CD8^+$ T cell responses, while treatment of an established trypanosome infection resulted in an increased $CD8^+$ T cell phenotype. These differences may be a result of the type of cytokines elicited at the time of either trypanosomes infection or ISMM administration. Bakheit *et al* (1990) and Olsson *et al* (1991) demonstrated that *T.*

brucei release a soluble factor that trigger $CD8^+$ T cells to produce IFN-γ. Sensitisation of $CD8^+$ T cells for the production of high levels of IFN-γ during experimental infections with *T. brucei* were also reported by Darji *et al* (1991).

In trypanosome control sheep, a non-significant increase in absolute $CD4^+$ T cells 7 to 14 days post infection was followed by a significant drop below normal levels 21 days later, while no significant changes were observed in the prophylactic group. In the trypanosome treated group a very small insignificant decrease in absolute $CD4^+$ T cells was observed 21 days after infection. In the BCG control group, 7 to 21 days after inoculation there was an increase in absolute $CD4^+$ T cells followed by a return to normal levels thereafter, but in the ISMM-BCG group there was little or no change in absolute $CD4^+$ T cells. These results indicate that ISMM prophylaxis prevented $CD4^+$ T cell losses by inhibiting non-specific polyclonal expansion (and accelerated cell death) probably in favour of antigen-specific expansion since trypanosome specific IgG antibody production was enhanced.

In general the ratio of $CD4^+$:$CD8^+$ T cells in the blood may be used to estimate lymphocyte function in clinical situation. An elevated $CD4^+$ T cell count implies increased lymphocyte reactivity as helper cells predominate, whereas a high $CD8^+$ T cell level implies depressed lymphocyte reactivity as a result of excessive suppressor activity (Tizard 1992). However, consequences of any changes in the ratio largely depends on the balance of cytokines produced by each cell types. In this study the ratio of $CD4^+$:$CD8^+$ T cells in the BCG control group increased 7 to 21 days after inoculation but returned to normal thereafter. In the ISMM-BCG group, an increase in $CD4^+$:$CD8^+$ T cells was observed throughout the study period. While in trypanosome control sheep an increase in the ratio was observed 7 to 14 days after infection, but decreased significantly below normal levels 21 days after infection, when an increase was being observed in the prophylactic group. These findings show increased levels of $CD4^+$ T cells in the prophylactic group, as compared to $CD8^+$ T cells which down regulate immune responses. This provides an explanation for high trypanosome specific IgG antibodies in the prophylactic compared to the control

group. The ratio of $CD4^+$:$CD8^+$ T cells in the treated group decreased largely because of an increase in $CD8^+$ T cells while in the control group it was a result of decreased $CD4^+$ T cells hence the probable reason for suppression of antibody levels in the treated group.

Contact between T cells and antigen presenting cells (APC) occurs through the T cell receptor (TCR) and MHC-peptide complex and involves a number of other accessory molecules (Tizard 1992). CD8 molecules associated with the TCR and the CD28 molecule on T cells bind to MHC-class I and B7 molecules on APCs respectively. CD4 molecules interact with MHC-class II molecules on APCs while interactions involving accessory molecules is similar to that occurring in $CD8^+$ T cells. These interactions result in the activation of T cells through signal transduction via the CD28 and the TCR. Activation of $CD4^+$ T cells leads to the induction of a high affinity IL-2 receptor (CD25), IL-2 and IFN-γ. The latter increases the expression of MHC class II on APCs, allowing more T cells to interact. IL-2 acts as a growth factor causing T cell proliferation and clonal expansion. IL-2 is not produced by $CD8^+$ T cells but is important for their expansion. The net effect of these events is to increase the number of antigen specific T cells, thereby increasing the chance of an earlier contact with the antigen in the body. Some of these activated cells may act locally at the site of antigen entry to help in antibody or cellular destruction of parasites, others will circulate and home at sites of antigen presence, and others will become memory cells. Both $CD4^+$ and $CD8^+$ T cells may participate in the elimination of trypanosomes by producing cytokines. For instance, IFN-γ converts monocytes into activated macrophages which are capable of eliminating trypanosomes by phagocytosis. Tumor necrotic factor (TNF) is another cytokine produced by activated T cells which has trypanocidal activity (Lucas *et al* 1993). The protective role of TNF was demonstrated by treating mice with soluble trypanosome components before infection with *T. brucei* to induce TNF production. One group of mice received anti-TNF antibodies at the same time soluble trypanosome components were administered. Results showed that parasite development occurred only in mice that received anti-TNF antibodies, implying that trypanosome induced TNF has a negative effect on trypanosome growth.

Absolute $\gamma\delta^+$ T cell numbers increased 7 to 21 after vaccination and returned to normal thereafter in the BCG control group. In the trypanosome control, a non-significant increase in absolute $\gamma\delta^+$ T cells was recorded 7 to 14 days after infection and was followed by a decrease below normal levels 21 days later. Treatment of trypanosome infection had little effect on absolute $\gamma\delta^+$ T cell responses. On the other hand, in the ISMM-BCG group, the absolute numbers of $\gamma\delta^+$ T cells showed a transient decrease 21 days after inoculation while in the trypanosome prophylaxis group a decrease 7 to 14 days after infection was observed. Therefore, ISMM appeared to have inhibited an increase in $\gamma\delta^+$ T cells that occurred between 7 and 14 days in control groups of both trypanosome infected and BCG vaccinated sheep. Other experiments in trypanosome infected sheep and cattle have demonstrated similar decreases in $\gamma\delta^+$ T cell phenotypes (Mwangi 1991; Williams *et al* 1991; Lutje *et al* 1995). However, the role of $\gamma\delta^+$ T cells in trypanosome infections is not clear. Flynn and Sileghem 1991 demonstrated that only $\gamma\delta^+$ T cells from *T. congolense* infected trypanotolerant N'dama and not susceptible Boran cattle proliferated *in vitro* suggesting that they may be involved in the control of the infection. Nevertheless, effects of ISMM prophylaxis on $\gamma\delta^+$ T cells seemed to be a result of the general inhibition of non-specific polyclonal lymhocyte proliferation rather than a specifically affecting these cells.

The PPD skin test is a diagnostic tool for estimating cell-mediated immune responses after exposure to *M. tuberculosis bovis* antigens. The reaction to the PPD skin test was not significantly different between the BCG control and ISMM-BCG groups, implying that despite modulation of certain aspects of the immune system, the actual response to challenge was not affected or that data was not sufficient to detect any significant difference. However, in trypanosome experiments, ISMM prophylaxis immunomodulated enhanced trypanosome specific IgG antibodies.

These results have relevant field implications related to the monitoring of ISMM prophylaxis. Currently prophylactic failure is generally considered to be a result of

either poor pharmacokinetics or trypanosome resistance through unknown mechanisms. However, these results demonstrate a mutual relationship between ISMM drug residues and the immune system in providing protection against trypanosome infections. Prophylaxis resulted in high trypanosome specific IgG antibodies in sheep while suppressing cellular responses in mice reduced the effectiveness of ISMM against *T. congolense* infection. These results also explain why certain animals may not be adequately protected even after careful administration of ISMM since the magnitude of immunomodulation depends on the status of the immune system at the time of drug administration. For instance, poor IL-12 and IFN-γ response might result in little or no protection while a heightened response may result in longer protection against trypanosomes. Thus, healthy animals would be expected to be protected for a longer period than those found with a poor state of the immune system at the time of administering ISMM. Based on these findings, it is important to minimise factors that suppress immune responses in order to obtain the maximum protection due to ISMM prophylaxis. That means stress factors need to be corrected and debilitating conditions such as worm infestation be addressed before ISMM is administered. Immunosuppressive agents such as corticosteroids should be contraindicated, while combination therapy with immunostimulants such as vitamin E and levamisole (an antihelminthic) may be advantageous (Tizard 1992). Levamisole stimulates phagocytic activity of macrophages and neutrophils. Its effects are greatest in animals with depressed T cell function, and it has little or no effect on the immune system of normal animals. Therefore, continuation of this work in the near future will involve the investigation of the combined use of ISMM and levamisole in addition to investigating further cytokine responses in ISMM treated animals.

In conclusion, this study has provided evidence that immunomodulation by ISMM enhances prophylaxis against *T. congolense* infection. Modulation of responses to BCG vaccination that were observed indicate that the mechanism of modulation is not antigen specific. ISMM activation of naive peripheral blood mononuclear cells *in vivo* for *in vitro* IFN-γ production after challenge with trypanosome antigens occurred suggesting that the mechanism of immunomodulation involves Th_1 cytokines with

IFN-γ and IL-12 feedback mechanism playing a major role. Unfortunately, these changes were not persistent to be used for monitoring protection. Further research is required to establish the best immunological marker that could be used for monitoring protection. Nevertheless, it is clear that ISMM provides a model for future prophylactic drug development if immunological parameters could be identified as markers of resistance to infection.

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APPENDICES

APPENDIX I
LIST OF MANUFACTURERS WITH MATERIALS SUPPLIED AND BUFFER SOLUTIONS

1. MANUFACTURERS AND MATERIALS SUPPLIED

Alpha laboratories Ltd, Hampshire, England: Microcentrifuge tubes and pipettes tips.

Becton Dickinson FACS Systems, Mountain View, CA, USA: Flow cytometer

Becton Dickinson, Meylan Cedex, France: 25cm^2 culture flasks, vacutainers, 96 well culture plates, needles and syringes.

BDH Ltd, Poole, England: Giemsa stain, hydrochloric acid, sodium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide

Bibby Sterilin Ltd, Staffordshire, England: Bijous and universals.

Commonwealth Serum Laboratories, 45 Popular Road, Parkville 3052, Victoria, Australia: Bovine γ interferon Test

Costar, Cambridge, USA: Pipetting reservoirs.

Department of Veterinary Pathology, University of Edinburgh: Monoclonal antibodies against CD4 (SBU-T4), CD5 (SBU-T1), CD8 (SBU-T8), γδ T cells (86D) and B cells (VPM30).

Department of Veterinary Physiology, University of Glasgow: ISMM-horse radish peroxidase conjugate and anti-ISMM rabbit serum.

Distillers Scotland: 5% carbon dioxide.

Dynatech: Immulon 4 96 well micro titre plates.

Exogen Ltd, Glasgow, Scotland: Glass wool

Genzyme, One Kendall Square, Cambridge, MA 02139-1562, USA: InterTest^{TM}-12X Total Mouse IL-12 ELISA Kit and InterTest^{TM}-12X Mouse IL-12p70 ELISA Kit and InterTest-γ^{TM} Mouse IFN-γ ELISA Kit.

Labsystems, Finland: Multichannel pipetters and Multiscan ELISA plate reader.

Life Technologies, Paisley, Scotland: IMDM (ISCOVES) RPMI 1640, MEM Eagle's with Earles salts, penicillin/streptomycin

May & Baker, Ltd, Dagenham, England: Isometamidium (Samorin®).

Millipore, Ireland: 0.22μ filters.

Neubauer, Germany: Haemocytometer.

Oxoid, Hampshire, England: PBS tablets.

Pharmacia, Uppsala, Sweden: Ficoll-Paque

Promega, Madison, USA: CellTitre 96™ Non-radioactive Cell Proliferation Assay Kit.

Sigma Chemical Co. Ltd, St. Louis, USA: Donor goat serum, L-glutamine, bathocuprionedsulphonic acid, mono-α-thioglycerol, adenosine, sodium pyruvate, foetal calf serum, and D-glucose, heparin, concanavalin A, sodium azide, bovine serum albumin, paraformaldehyde, EDTA, tween 20,

The Binding Site, Birmingham, England: FITC- mouse- anti-IgG_1, IgG_{2a} and IgM conjugates.

Watman, Maidstone, England: Diethyl cellulose-52 (DE52).

Wild Leitz GMBH, Germany: Light microscope

2. BUFFER SOLUTIONS

Phosphate buffered saline (PBS pH 7.4): Prepared by either dissolving 1 PBS tablet per 100ml of distilled deionised water or making up the following; 4g sodium chloride (NaCl) (0.8% w/v), 0.74g sodium hydrogen phosphate (Na_2HPO_4) (0.148% w/v) and 0.215g potassium dihydrogen phosphate (KH_2PO_4) (0.043% w/v) and made up to 500ml with distilled deionised water.

1% paraformaldehyde fixative for PBMCs: 50ml PBS were heated to 60°C and 1g paraformaldehyde added followed by the addition drop by drop of 1M NaOH until paraformaldehyde dissolved. The solution was made up to 100ml with PBS and cooled under tap water and pH adjusted to 7.0 before storing at 4°C for up to a week.

Working strength of 0.1% paraformaldehyde was prepared by taking 10ml of 1% paraformaldehyde and making it up to 100ml with PBS.

PBMC phenotyping wash buffer: 0.1% sodium azide, 1% bovine serum albumin and 20U/ml heparin in PBS.

ELISA diluent/wash buffer: 0.05% tween 20 in PBS (PBST pH 7.4): e.g. 0.25ml tween 20 in 500ml PBS.

ELISA coating buffer: Carbonate/bicarbonate (pH9.6). consisting of 0.159% (w/v) sodium carbonate, 0.293% w/v sodium bicarbonate and made up to 100ml with distilled water.

APPENDIX II
RECTAL TEMPERATURES AND HAEMOTOLOGICAL RESULT TABLES

Table A1(a): Rectal temperatures (°C) for the Control group following primary infection with *T. congolense*

Days post infection	(a). CONTROL GROUP			
	2BF	3WS	1499	2386
0	40.0	39.8	39.7	40.0
1	39.8	40.0	39.5	39.8
2	40.2	39.9	39.9	39.9
3	40.1	39.8	39.8	40.1
4	41.0	42.1	40.8	40.9
5	40.4	40.2	40.8	40.3
6	40.2	40.2	40.4	40.4
7	40.1	40.6	40.5	40.3
8	40.1	40.2	39.8	40.0
9	40.0	40.3	40.2	40.3
10	41.0	40.8	40.2	40.0
11	40.9	40.5	40.3	39.8
12	40.9	40.6	40.3	40.0
13	40.7	40.5	40.4	40.2
14	40.2	41.0	40.9	41.0
15	40.4	40.4	40.8	40.9
16	40.2	39.9	40.6	40.5
17	40.4	39.8	40.1	40.1
18	40.5	40.2	40.4	40.3
19	40.4	40.1	40.5	40.2
20	40.3	40.0	40.5	40.2
21	40.1	40.2	40.3	40.2
22	40.3	40.0	40.3	40.0
23	40.1	39.8	40.4	39.9
24	40.2	40.0	40.6	40.2
25	40.2	40.1	40.7	40.6
26	40.0	40.3	40.5	40.4
27	39.9	40.1	40.5	40.1
28	40.0	39.9	39.8	40.3
29	40.0	39.9	39.9	40.1
30	40.0	39.9	40.2	40.1
31	40.8	39.9	39.6	40.4
32	40.1	39.7	39.4	40.2
33	40.2	40.4	39.6	40.2
34	40.1	40.4	39.8	40.4
35	40.2	40.2	40.3	40.5
36	40.2	40.3	40.1	40.7
37	40.0	40.0	39.7	40.3
38	40.1	39.9	39.5	40.3

~ *All animals infected on day zero*

Table A1(b): Rectal temperatures (°C) for the Prophylactic group following primary infection with *T. congolense*

Days post infection	(b). PROPHYLACTIC GROUP			
	1618	1746	1826	1895
0	39.7	40.0	39.8	40.0
1	39.8	39.8	40.0	39.8
2	39.7	39.6	39.9	39.9
3	39.9	40.1	39.8	40.1
4	40.1	39.8	39.6	39.5
5	40.2	40.1	39.6	39.6
6	39.8	40.2	39.9	39.6
7	39.9	40.1	39.8	39.8
8	39.7	40.1	39.8	40.0
9	40.2	40.0	39.6	39.8
10	40.0	39.7	40.0	39.7
11	39.9	39.8	39.8	39.8
12	40.0	39.9	39.7	39.7
13	40.1	39.7	39.9	40.1
14	40.2	39.8	40.0	39.5
15	39.5	40.1	39.7	39.7
16	39.8	40.0	39.9	39.9
17	39.9	39.5	39.8	39.8
18	39.1	39.6	40.0	39.8
19	40.2	39.6	40.1	39.8
20	39.6	39.7	40.0	39.7
21	39.8	40.1	39.8	39.5
22	39.3	39.8	40.0	40.0
23	39.8	39.8	39.8	39.9
24	39.5	40.0	39.9	39.8
25	40.1	39.9	40.1	39.7
26	39.6	39.7	39.6	39.6
27	39.8	39.9	39.5	39.9
28	40.1	40.0	39.9	39.9
29	40.0	39.8	39.6	40.1
30	39.8	39.8	39.9	40.0
31	39.7	39.6	39.9	39.6
32	39.0	39.9	39.7	39.8
33	39.3	39.8	39.7	39.6
34	39.9	40.1	39.6	39.7
35	39.8	39.7	39.8	39.8
36	39.9	39.6	39.7	39.8
37	39.4	40.0	40.0	39.9
38	39.7	39.8	39.9	39.6

~ All animals infected on day zero

~ Prophylaxis done four and half months before infection

Table A1(c): Rectal temperature (°C) for the Treated group following primary infection with *T. congolense*

Days post infection	(c). TREATED GROUP			
	1736	1863	2381	2383
0	39.7	39.9	39.7	39.6
1	39.8	39.6	39.6	39.5
2	39.7	39.7	39.9	39.7
3	39.9	39.9	39.9	39.8
4	40.1	41.1	40.8	40.8
5	40.2	39.8	40.6	40.4
6	40.3	40.4	40.1	40.3
7	39.9	40.0	40.6	40.9
8	40.7	40.3	40.5	40.4
9	40.4	40.1	40.4	40.8
10	40.0	40.6	40.4	40.7
11	39.9	41.0	40.4	41.0
12	40.0	41.0	40.3	40.9
13	40.2	41.0	40.3	40.7
14	40.2	40.3	40.2	40.8
15	40.8	40.3	40.6	40.6
16	40.7	40.4	41.0	41.2
17	40.5	40.6	40.0	40.6
18	40.1	40.1	40.3	40.2
19	40.2	40.1	40.0	40.5
20	40.5	40.3	40.4	40.8
21	40.6	40.3	40.7	40.5
22	40.7	40.5	40.4	40.7
23	40.2	40.1	40.6	40.7
24	40.4	40.5	40.0	41.6
25	40.1	40.3	40.4	41.0
26	40.0	40.6	40.4	40.6
27	39.8	40.6	40.6	40.2
28	40.1	40.7	40.8	39.7
29	40.4	40.8	41.6	39.5
30	40.6	40.4	40.7	39.5
31	41.0	40.6	40.9	40.8
32	40.8	40.6	41.1	40.6
33	40.5	40.8	41.2	40.6
34	40.0	41.0	41.8	40.7
35	40.2	41.3	41.8	40.4
36	40.4	41.2	41.5	40.3
37	40.8	40.6	40.7	40.1
38	41.0	40.1	40.4	40.0

~ *All animals infected on day zero*

~ *Treatment done 14 days post infection*

Table A.2: Packed cell volume (%) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	32	32	38	32	32
-7	32	33	38	32	33
0	32	32	37	32	32
7	29	31	37	31	31
14	30	30	35	31	31
17	26	26	30	28	27
21	30	28	30	29	30
24	24	25	29	27	26
28	28	28	30	28	28
31	25	24	25	28	25
35	22	23	24	29	24
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	41	38	38	36	38
-7	41	36	38	36	37
0	40	38	39	37	39
7	41	37	37	38	38
14	40	38	38	36	38
17	41	36	39	38	39
21	42	38	38	37	38
24	41	37	37	37	37
28	41	39	38	36	39
31	40	38	38	38	38
35	42	37	39	36	38
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	34	35	37	33	35
-7	34	36	37	34	35
0	35	36	38	34	36
7	33	34	36	32	34
14	31	31	33	31	31
17	31	28	31	27	30
21	33	27	30	33	32
24	33	31	35	32	33
28	34	31	34	33	34
31	32	31	34	34	33
35	34	34	36	30	34

~ *All animals infected on day zero*

~ *Prophylaxis done four and half months before infection*

~ *Treatment done 14 days post infection*

Table A3: Red Blood Cell count (x10^9/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	11.3	11.1	12.7	12.1	11.7
-7	11.4	10.6	12.2	11.6	11.5
0	10.9	10.8	12.5	11.9	11.4
7	10.2	10.7	12.3	11.7	11.2
14	12.6	11.1	14.0	11.9	12.2
21	9.9	8.9	10.1	10.6	10.0
28	9.1	8.7	9.2	10.4	9.2
35	9.9	7.7	9.1	11.0	9.5
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	13.4	12.3	12.5	11.9	12.4
-7	13.6	11.6	12.0	12.7	12.4
0	14.0	11.9	12.7	12.3	12.5
7	15.7	12.5	13.2	12.8	13.0
14	14.3	11.0	12.0	11.4	11.7
21	14.8	11.9	11.8	12.1	12.0
28	14.7	12.2	12.0	12.2	12.2
35	15.3	11.9	11.8	11.9	11.9
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	12.1	13.5	12.8	12.4	12.6
-7	11.9	13.9	13.5	11.9	12.7
0	11.5	13.4	13.6	12.4	12.9
7	11.3	14.0	12.8	12.5	12.6
14	12.0	13.1	13.8	11.5	12.5
21	12.6	10.6	13.9	11.5	12.0
28	13.1	10.9	12.2	12.6	12.4
35	15.9	13.8	12.8	10.7	13.3

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table A4: White blood cell count (x10^6/ml) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	7.2	7.0	7.3	6.8	7.1
-7	7.4	7.3	7.1	6.9	7.2
0	7.4	7.4	7.0	7.0	7.2
7	8.0	10.1	10.1	9.7	9.9
14	9.6	11.2	12.9	12.2	11.7
21	9.7	11.6	13.7	10.1	10.9
28	11.0	11.4	13.3	9.5	11.2
35	6.6	11.2	14.2	10.8	11.0
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	6.1	13.0	8.7	6.8	7.8
-7	5.9	12.9	8.5	7.1	7.8
0	6.3	12.4	8.6	6.9	7.8
7	7.1	11.2	9.1	6.8	8.1
14	8.2	12.4	9.7	6.8	9.0
21	8.8	17.0	8.8	5.5	8.8
28	6.9	13.0	9.3	6.2	8.1
35	6.5	12.7	8.9	7.0	8.0
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	11.9	12.0	8.0	6.4	10.0
-7	12.4	11.7	8.4	6.7	10.1
0	12.2	11.8	8.3	6.5	10.1
7	11.5	11.8	8.2	7.6	9.9
14	12.1	15.0	10.4	10.4	11.3
21	17.2	16.6	10.9	10.5	13.8
28	15.6	13.9	8.1	8.8	11.4
35	17.4	15.1	8.5	8.0	11.8

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table A5: Absolute Lymphocytes (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	5.0	4.8	4.7	4.8	4.8
-7	5.1	4.7	4.4	4.6	4.7
0	5.4	5.0	4.5	4.7	4.8
7	5.7	6.7	6.2	6.8	6.4
14	7.2	8.3	8.9	9.8	8.6
21	7.2	10.8	12.2	9.2	10.0
28	7.2	9.5	11.2	8.0	8.7
35	5.0	10.1	11.1	9.4	9.7
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	4.2	9.9	6.5	4.6	5.5
-7	4.2	10.1	6.8	5.0	5.9
0	4.4	9.3	6.6	4.8	5.7
7	5.0	8.6	6.9	5.2	6.1
14	6.2	9.9	6.8	4.8	6.5
21	6.5	14.3	6.6	4.1	6.6
28	5.0	10.0	6.5	4.4	5.7
35	4.7	9.9	6.6	5.1	5.9
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	6.3	8.4	4.9	4.5	5.6
-7	7.1	7.6	4.8	5.0	6.0
0	6.6	8.5	4.9	4.7	5.7
7	6.4	8.3	4.9	5.7	6.1
14	7.7	11.0	6.7	8.6	8.2
21	12.0	13.5	6.7	8.1	10.1
28	12.2	11.1	6.2	8.1	9.6
35	13.9	10.4	6.8	6.6	8.6

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table A6: Absolute Monocytes (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	0.2	0.1	0.1	0.2	0.2
-7	0.3	0.2	0.3	0.3	0.3
0	0.3	0.1	0.1	0.4	0.2
7	0.4	0.2	0.4	0.3	0.3
14	0.6	0.8	1.2	0.6	0.7
21	0.3	0.2	0.4	0.1	0.3
28	0.9	0.3	0.3	0.1	0.3
35	0.8	0.2	0.4	0.6	0.5
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	0.4	0.5	0.3	0.3	0.4
-7	0.2	0.4	0.2	0.2	0.2
0	0.3	0.4	0.3	0.3	0.3
7	0.3	0.3	0.3	0.3	0.3
14	0.2	0.2	0.7	0.3	0.3
21	0.3	0.3	0.4	0.2	0.3
28	0.1	0.7	0.7	0.4	0.5
35	0.1	0.3	0.4	0.4	0.3
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	0.6	0.5	0.4	0.2	0.4
-7	0.6	0.7	0.5	0.2	0.6
0	0.5	0.4	0.3	0.2	0.3
7	0.6	0.2	0.5	0.2	0.4
14	1.0	0.9	0.9	0.5	0.9
21	0.5	0.5	1.0	0.8	0.6
28	0.5	0.3	0.6	0.3	0.4
35	0.3	0.8	0.3	0.3	0.3

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table A7: Absolute Neutrophils (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	1.7	1.9	2.2	1.6	1.8
-7	1.7	2.1	2.2	1.7	1.9
0	1.5	2.1	2.2	1.6	1.9
7	1.7	2.9	3.2	2.3	2.6
14	1.6	1.9	2.6	1.5	1.8
21	2.0	0.5	1.0	0.6	0.8
28	2.5	1.5	1.7	1.1	1.6
35	7.9	6.7	2.6	0.5	4.6
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	1.5	2.2	1.5	1.6	1.6
-7	1.4	2.1	1.3	1.6	1.5
0	1.4	2.0	1.4	1.6	1.5
7	1.6	1.9	1.5	1.1	1.6
14	1.6	1.9	1.9	1.4	1.7
21	1.8	2.0	1.6	0.9	1.7
28	1.7	2.1	1.8	1.2	1.7
35	1.4	2.1	1.6	1.3	1.5
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	4.5	2.8	2.5	1.4	2.6
-7	4.3	3.2	2.8	1.5	3.0
0	4.6	2.7	2.8	1.4	2.8
7	4.0	3.0	2.6	1.6	2.8
14	3.3	2.9	2.5	1.0	2.7
21	4.6	2.3	3.2	1.6	2.7
28	2.8	2.4	1.1	0.3	1.7
35	2.6	3.3	1.2	1.0	1.9

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table A8: Absolute Eosinophils (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	0.3	0.2	0.2	0.3	0.2
-7	0.3	0.2	0.1	0.2	0.2
0	0.2	0.1	0.2	0.3	0.2
7	0.2	0.3	0.2	0.3	0.3
14	0.2	0.2	0.3	0.4	0.2
21	0.1	0.1	0.1	0.2	0.1
28	0.4	0.1	0.1	0.3	0.2
35	0.1	0.2	0.1	0.2	0.2
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	1746	1826	1895	Median
-14	0.1	0.4	0.3	0.3	0.3
-7	0.2	0.4	0.3	0.3	0.3
0	0.2	0.2	0.3	0.2	0.2
7	0.1	0.3	0.4	0.2	0.3
14	0.2	0.4	0.3	0.3	0.3
21	0.3	0.3	0.3	0.2	0.3
28	0.1	0.3	0.3	0.2	0.2
35	0.2	0.3	0.4	0.2	0.2
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	3WS	1499	2386	Median
-14	0.5	0.4	0.2	0.2	0.3
-7	0.4	0.2	0.3	0.1	0.3
0	0.5	0.2	0.2	0.2	0.2
7	0.5	0.4	0.2	0.1	0.3
14	0.1	0.3	0.3	0.2	0.3
21	0.0	0.3	0.1	0.1	0.1
28	0.2	0.1	0.2	0.1	0.1
35	0.5	0.6	0.3	0.2	0.4

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

APPENDIX III
RAW DATA FOR *IN VITRO* AND *IN VIVO* EFFICACY OF ISMM

TABLE B1: ISMM PLASMA PROFILE (ng/ml)

Days pre/post ISMM	SHEEP NUMBER				
administration	1618	1746	1826	1895	Median
1	3.21	6.86	23.32	40.52	15.09
2	1.21	3.16	12.85	20.94	8.01
3	1.33	3.14	4.95	14.80	4.05
4	1.19	1.99	2.58	10.13	2.29
7	1.08	1.33	1.58	6.89	1.46
14	1.05	1.12	1.35	4.52	1.24
21	1.04	1.10	1.16	3.00	1.13

TABLE B2: SENSITIVITY OF TRYPANOSOMES TO ISMM *IN VITRO*

ISMM conc.	% GROWTH INHIBITION	
(ng/ml)	Strain 1467	Strain 1881
0	0	0
1	22	62
10	45	69
100	65	85
1000	72	90
10000	74	92

TABLE B3: PREPATENT PERIOD (DAYS) FOR MICE WITH DIFFERENT IMMUNE STATUSES INFECTED WITH *T. CONGOLENSE*

Mouse #	Control	Hydrocort.	ISMM	Hydroco.+ISMM
1	3	4	7	4
2	3	3	6	3
3	3	3	7	2
4	4	2	8	3
5	3	4	7	3
Median	**3**	**3**	**7**	**3**

TABLE B4: SURVIVAL TIME (DAYS) FOR MICE WITH DIFFERENT IMMUNE STATUSES INFECTED WITH *T. CONGOLENSE*

Mouse #	Control	Hydrocort.	ISMM	Hydroco.+ISMM
1	15	16	24	20
2	18	22	21	17
3	19	19	26	15
4	23	14	27	28
5	17	18	29	16
Median	**18**	**18**	**26**	**17**

APPENDIX IV
TABLES OF RESULTS FOR PBMC PHENOTYPES

Table C1.1: Percentage of B-cells following primary infection with *T.congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	39.4	50.3	61.2	40.1	45.2
-7	36.8	48.7	60.1	37.6	43.2
0	42.4	52.8	59.2	39.9	47.6
7	39.4	46.4	54.0	34.6	42.9
14	60.0	57.0	74.8	57.2	58.6
21	82.7	78.0	78.9	55.3	78.5
28	46.9	53.8	70.6	34.5	50.4
35	51.5	37.2	63.1	39.6	45.6
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	40.3	41.6	44.1	46.5	42.9
-7	39.9	44.1	45.4	49.5	44.8
0	39.2	40.5	41.7	45.3	41.1
7	41.6	44.7	47.1	49.1	45.9
14	53.5	51.8	52.8	53.5	53.2
21	45.2	41.1	55.2	58.6	50.2
28	47.1	43.9	47.4	46.9	47.0
35	41.3	44.2	44.5	47.4	44.4
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	37.4	49.8	41.6	40.2	40.9
-7	35.7	53.3	44.0	39.8	41.9
0	39.7	51.9	41.2	44.2	42.7
7	37.9	50.2	39.5	41.8	40.7
14	66.7	60.9	60.9	66.4	63.7
21	67.3	68.2	61.4	68.0	67.7
28	40.7	53.8	51.7	45.0	48.4
35	19.2	41.5	41.3	44.2	41.4

~ All animals infected on day zero
~ Prophylaxis done four and half months before infection
- Treatment done 11 days post infection

Table C1.2: Absolute B cells (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	1.98	2.39	2.90	1.91	2.19
-7	1.88	2.31	2.64	1.74	2.10
0	2.29	2.62	2.65	1.87	2.46
7	2.24	3.09	3.33	2.35	2.72
14	4.32	4.72	6.66	5.58	5.15
21	5.94	8.42	9.62	5.08	7.18
28	3.35	5.09	7.89	2.75	4.22
35	2.55	3.75	6.99	3.72	3.74
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	1746	1826	1895	Median
-14	1.67	4.1	2.88	2.12	2.50
-7	1.67	4.44	3.09	2.46	2.78
0	1.73	3.77	2.76	2.16	2.46
7	2.1	3.85	3.26	2.57	2.92
14	3.33	5.14	3.58	2.55	3.46
21	2.94	5.87	3.64	2.41	3.29
28	2.34	4.39	3.08	2.06	2.71
35	1.96	4.38	2.93	2.42	2.68
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	3WS	1499	2386	Median
-14	2.26	4.18	2.03	1.83	2.15
-7	2.52	4.05	2.11	1.97	2.32
0	2.62	4.41	2.02	2.07	2.35
7	2.44	4.15	1.94	2.38	2.41
14	5.16	6.67	4.06	5.73	4.06
21	8.10	9.17	4.08	5.49	6.80
28	4.95	5.98	3.23	3.64	4.30
35	2.68	4.32	2.81	2.90	2.86

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C2.1: Percentage of $CD5^+$ T-cells following primary infection with *T.congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	51.4	41.2	42.1	54.6	46.8
-7	50.1	43.0	44.6	55.9	47.4
0	53.5	44.1	40.1	58.2	48.8
7	56.5	47.6	43.7	56.2	51.9
14	35.1	34.8	27.8	36.0	35.0
21	18.5	19.4	20.6	33.5	20.0
28	33.4	33.8	25.6	41.5	33.6
35	33.6	36.1	26.0	38.6	34.9
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	52.2	47.9	46.6	45.1	47.3
-7	54.1	49.9	48.6	44.5	49.3
0	53.8	52.5	52.3	48.7	52.4
7	51.4	46.5	45.9	43.9	46.2
14	39.5	41.2	41.2	39.5	40.4
21	47.8	55.9	37.7	36.4	42.8
28	45.9	50.1	46.6	49.1	47.9
35	52.7	49.8	49.5	44.6	49.7
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	42.5	49.6	53.7	45.3	47.5
-7	40.7	52.2	54.7	48.4	50.3
0	43.6	47.8	52.5	43.1	45.7
7	45.7	50.2	55.1	46.3	48.3
14	25.0	32.5	38.3	31.1	31.8
21	21.8	27.7	34.6	24.3	26.0
28	49.1	33.9	37.1	36.7	36.9
35	29.5	33.2	42.1	34.6	33.9

~ All animals infected on day zero
~ Prophylaxis done four and half months before infection
~ Treatment done 14 days post infection

Table C2.2 Absolute $CD5^+$ T cells ($x10^6$/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	2.59	1.96	2.00	2.29	2.15
-7	2.56	2.04	1.96	2.28	2.16
0	2.89	2.19	1.80	2.40	2.30
7	9.95	3.17	2.69	4.91	4.04
14	2.53	2.88	2.47	2.85	2.69
21	1.33	2.09	2.51	2.25	2.17
28	2.39	3.20	2.86	2.94	2.90
35	1.66	3.64	2.88	2.95	2.92
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	2.17	4.73	3.04	2.06	2.61
-7	2.27	5.02	3.30	2.21	2.79
0	2.37	4.88	3.46	2.34	2.92
7	2.59	4.01	3.18	2.30	2.89
14	2.36	4.09	2.80	1.88	2.58
21	3.11	7.98	2.49	1.50	2.80
28	2.28	5.02	3.03	2.16	2.66
35	2.50	4.94	3.26	2.28	2.88
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	2.68	4.17	2.62	2.06	2.65
-7	2.88	3.97	2.62	2.40	2.75
0	2.87	4.06	2.58	2.02	2.73
7	2.94	4.15	2.71	2.64	2.83
14	1.94	3.56	2.55	2.68	2.62
21	2.62	3.73	2.30	1.96	2.46
28	5.98	3.77	2.32	2.97	3.37
35	4.11	3.46	2.86	2.27	3.16

~ All animals infected on day zero
~ Prophylaxis done four and half months before infection
~ Treatment done 14 days post infection

Table C3.1 Percentage of $\gamma\delta^+$ T-cells following primary infection with *T.congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	13.3	15.8	8.1	16.8	14.6
-7	12.4	16.2	9.3	17.4	14.3
0	14.0	17.4	8.4	17.8	15.7
7	14.3	16.5	8.2	18.0	15.4
14	9.3	11.5	5.0	12.0	10.4
21	1.2	3.8	2.7	3.2	3.0
28	11.4	8.7	3.6	12.0	10.1
35	11.0	10.1	3.8	8.4	9.3
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	12.5	15.3	14.7	19.6	15.0
-7	13.5	14.8	13.0	18.7	14.2
0	12.1	15.6	16.6	21.9	16.1
7	9.7	10.0	9.8	18.9	9.9
14	0.4	4.9	5.0	14.0	5.0
21	13.9	17.2	11.6	14.1	14.0
28	12.9	16.0	16.5	23.9	16.3
35	15.5	14.0	19.2	17.4	16.5
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	13.9	16.9	9.1	10.8	12.4
-7	14.8	17.8	9.8	11.4	13.1
0	14.6	17.4	10.1	12.3	13.5
7	15.8	18.3	8.9	11.0	13.4
14	4.9	9.5	6.6	6.8	6.7
21	4.0	4.3	5.9	7.4	5.1
28	6.5	14.3	8.6	7.9	8.3
35	4.7	15.0	8.1	10.3	9.2

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C3.2 Absolute $\gamma\delta^+$ T cells (x10^6/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	0.67	0.75	0.38	0.80	0.71
-7	0.63	0.77	0.41	0.80	0.70
0	0.76	0.86	0.38	0.83	0.80
7	1.03	1.10	0.51	1.22	1.07
14	0.67	0.95	0.44	1.17	0.81
21	0.09	0.41	0.33	0.29	0.31
28	0.82	0.82	0.40	0.96	0.82
35	0.54	1.02	0.42	0.79	0.67
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	0.52	1.51	0.96	0.89	0.93
-7	0.56	1.49	0.88	0.93	0.91
0	0.53	1.45	1.10	1.04	1.07
7	0.49	0.86	0.68	0.99	0.77
14	0.02	0.49	0.34	0.67	0.42
21	0.90	1.21	0.76	0.58	0.83
28	0.64	1.60	1.07	1.05	1.06
35	0.73	1.39	1.26	0.89	1.08
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	0.88	1.42	0.44	0.49	0.69
-7	1.05	1.35	0.47	0.56	0.81
0	0.96	1.48	0.49	0.58	0.77
7	1.02	1.51	0.44	0.63	0.83
14	0.38	1.04	0.44	0.59	0.52
21	0.48	0.58	0.39	0.60	0.53
28	0.79	1.59	0.54	0.64	0.72
35	0.65	1.56	0.55	0.68	0.67

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C4.1 Percentage of $CD8^+$ T-cells following primary infection with *T.congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	13.4	5.8	5.5	16.4	9.6
-7	14.1	5.6	6.5	16.0	10.3
0	13.5	6.2	5.3	15.5	9.9
7	12.4	5.2	5.0	12.8	8.8
14	5.9	3.2	1.7	6.4	4.6
21	4.3	2.2	2.8	10.5	3.6
28	8.1	5.5	1.9	16.5	6.8
35	5.6	6.2	3.0	8.5	5.9
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	11.9	13.4	14.2	9.2	12.7
-7	12.6	12.6	13.6	8.7	12.6
0	13.3	14.2	13.9	10.0	13.6
7	12.3	14.5	13.4	7.9	12.9
14	13.2	15.0	15.3	9.1	14.1
21	6.8	10.2	5.3	5.7	6.3
28	7.2	13.8	7.7	9.2	8.5
35	10.6	13.0	8.0	9.9	10.3
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	5.7	7.8	12.1	11.6	9.7
-7	6.5	7.7	11.7	12.6	9.7
0	5.8	8.4	12.6	11.4	9.9
7	6.9	8.1	12.7	11.9	10.0
14	4.1	4.9	6.8	4.0	4.5
21	10.0	8.6	8.8	8.5	8.7
28	9.9	7.7	9.7	11.1	9.8
35	6.9	5.7	10.7	9.8	8.4

~ All animals infected on day zero
~ Prophylaxis done four and half months before infection
~ Treatment done 14 days post infection

Table C4.2 Absolute $CD8^+$ T cells ($x10^6$/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	1863	2381	2383	Median
-14	0.68	0.28	0.26	0.78	0.48
-7	0.72	0.26	0.29	0.74	0.51
0	0.73	0.31	0.24	0.73	0.52
7	0.70	0.35	0.31	0.79	0.53
14	0.42	0.26	0.15	0.62	0.34
21	0.31	0.24	0.34	0.96	0.33
28	0.58	0.52	0.21	1.32	0.55
35	0.28	0.62	0.33	0.80	0.48
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	1746	1826	1895	Median
-14	0.49	1.32	0.92	0.42	0.71
-7	0.53	1.27	0.92	0.43	0.73
0	0.59	1.32	0.92	0.48	0.76
7	0.62	1.25	0.93	0.41	0.78
14	0.82	1.49	1.03	0.43	0.93
21	0.44	1.46	0.35	0.23	0.40
28	0.36	1.38	0.50	0.40	0.45
35	0.50	1.29	0.53	0.21	0.52
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	3WS	1499	2386	Median
-14	0.36	0.66	0.59	0.53	0.56
-7	0.46	0.65	0.56	0.62	0.59
0	0.38	0.71	0.62	0.53	0.58
7	0.44	0.67	0.62	0.68	0.65
14	0.32	0.54	0.45	0.34	0.40
21	1.21	1.16	0.58	0.69	0.93
28	1.20	0.86	0.60	0.90	0.88
35	0.96	0.59	0.73	0.64	0.69

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C5.1 Percentage of $CD4^+$ T-cells following primary infection with *T.congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	23.8	14.3	16.1	24.2	20.0
-7	24.6	15.2	16.8	23.8	20.3
0	22.9	13.7	17.6	24.3	20.3
7	23.9	15.4	17.4	23.3	20.4
14	13.4	13.3	9.4	14.1	13.4
21	2.1	2.7	3.9	10.0	3.3
28	8.9	12.7	6.9	17.0	10.8
35	11.1	16.3	6.7	15.2	13.2
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	29.3	21.2	21.6	16.5	21.4
-7	28.0	20.2	22.0	17.1	21.1
0	28.4	22.7	21.8	16.8	22.3
7	29.4	22.0	22.7	17.1	22.4
14	25.9	21.3	20.9	16.4	21.1
21	27.1	23.0	20.8	16.6	21.9
28	25.8	20.3	22.4	16.0	21.4
35	26.6	22.8	22.3	17.3	22.6
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	13.2	11.4	23.3	17.1	15.2
-7	13.9	12.2	23.7	16.4	15.2
0	14.9	11.8	24.2	17.6	16.3
7	14.1	11.6	24.7	18.0	16.1
14	7.8	11.0	12.5	11.9	11.5
21	3.5	4.0	10.3	4.8	4.4
28	13.8	11.6	13.1	12.9	13.0
35	8.2	10.6	18.8	13.5	12.1

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C5.2 Absolute $CD4^+$ T cells ($x10^6$/ml blood) following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	1.20	0.68	0.76	1.15	0.96
-7	1.24	0.72	0.74	1.10	0.92
0	1.22	0.68	0.79	1.14	0.97
7	1.34	1.03	1.07	1.58	1.21
14	0.96	1.10	0.84	1.38	1.03
21	0.15	0.29	0.48	0.92	0.39
28	0.64	1.20	0.77	1.36	0.99
35	0.55	1.64	0.74	1.43	1.09
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	1.22	2.09	1.41	0.75	1.32
-7	1.17	2.03	1.50	0.85	1.34
0	1.25	2.11	1.44	0.80	1.35
7	1.48	1.90	1.57	0.90	1.53
14	1.61	2.11	1.42	0.78	1.52
21	1.76	3.28	1.37	0.68	1.57
28	1.28	2.03	1.46	0.70	1.37
35	1.26	2.26	1.47	0.88	1.37
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	0.83	0.96	1.14	0.78	0.90
-7	0.98	0.93	1.14	0.81	0.96
0	0.98	1.00	1.18	0.82	0.99
7	0.91	0.96	1.22	1.03	1.00
14	0.60	1.20	0.83	1.03	0.93
21	0.42	0.54	0.68	0.39	0.48
28	1.68	1.29	0.82	1.04	1.17
35	1.14	1.10	1.28	0.88	1.12

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

Table C6.0 $CD4^+$:$CD8^+$ ratios following primary infection with *T. congolense*

Days pre/post infection	(a). CONTROL GROUP				
	1736	**1863**	**2381**	**2383**	**Median**
-14	1.76	2.43	2.92	1.47	2.10
-7	1.72	2.77	2.55	1.49	2.14
0	1.67	2.19	3.29	1.56	1.93
7	1.91	2.94	3.45	2.00	2.47
14	2.28	4.23	5.60	2.22	3.26
21	0.48	1.21	1.41	0.96	1.09
28	1.10	2.31	3.67	1.03	1.71
35	1.96	2.64	2.24	1.79	2.10
Days pre/post infection	**(b). PROPHYLACTIC GROUP**				
	1618	**1746**	**1826**	**1895**	**Median**
-14	2.49	1.58	1.53	1.78	1.68
-7	2.21	1.60	1.63	1.98	1.81
0	2.12	1.60	1.56	1.67	1.64
7	2.39	1.52	1.69	2.20	1.95
14	1.96	1.42	1.38	1.81	1.62
21	4.00	2.25	3.91	2.96	3.44
28	3.56	1.47	2.92	1.75	2.34
35	2.52	1.75	2.77	4.19	2.65
Days pre/post infection	**(c). TREATED GROUP**				
	2BF	**3WS**	**1499**	**2386**	**Median**
-14	2.30	1.45	1.93	1.47	1.70
-7	2.13	1.43	2.04	1.31	1.74
0	2.58	1.41	1.90	1.55	1.73
7	2.07	1.43	1.97	1.51	1.74
14	1.88	2.22	1.84	3.03	2.05
21	0.35	0.46	1.17	0.56	0.51
28	1.40	1.50	1.37	1.16	1.39
35	1.19	1.86	1.75	1.38	1.57

~ *All animals infected on day zero*
~ *Prophylaxis done four and half months before infection*
~ *Treatment done 14 days post infection*

FIGURE C1: REPRESENTATIVE B CELL HISTOGRAMS FOR THE CONTROL GROUP(Sheep 1736)

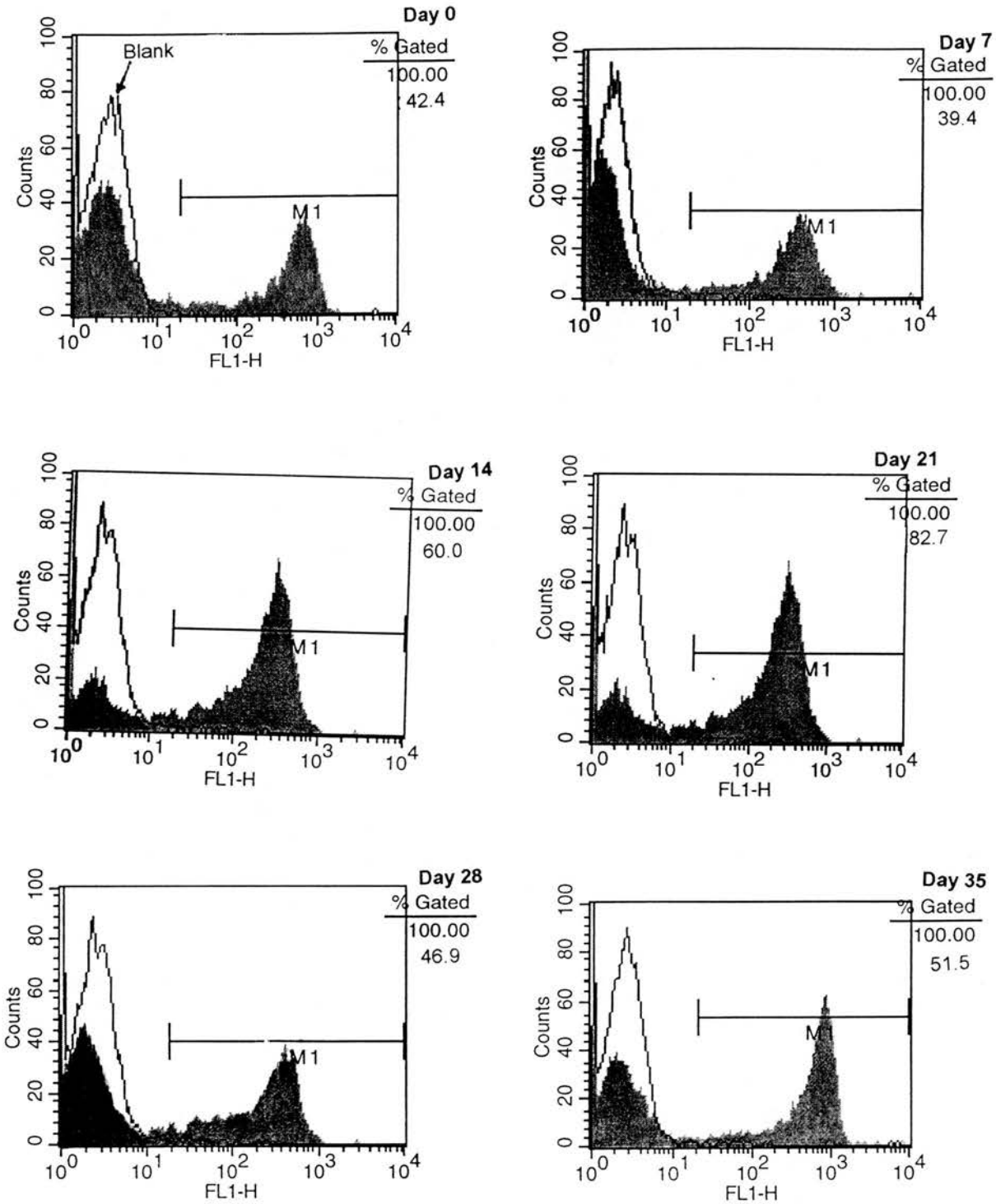


FIGURE C2: REPRESENTATIVE B CELL HISTOGRAMS FOR THE PROPHYLACTIC GROUP(Sheep 1826)

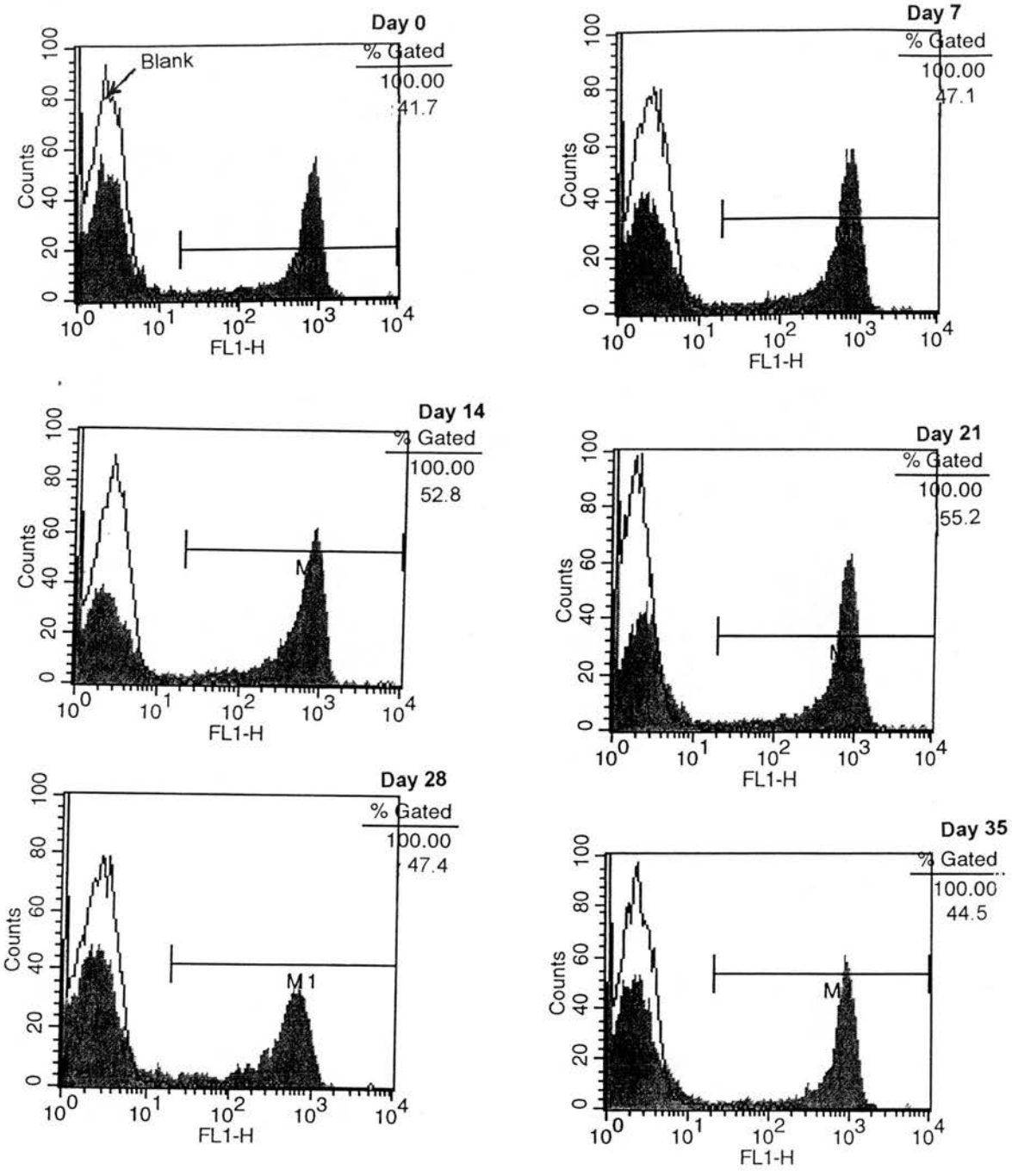


FIGURE C3: REPRESENTATIVE B CELL HISTOGRAMS FOR THE TREATED GROUP(Sheep 3WS)

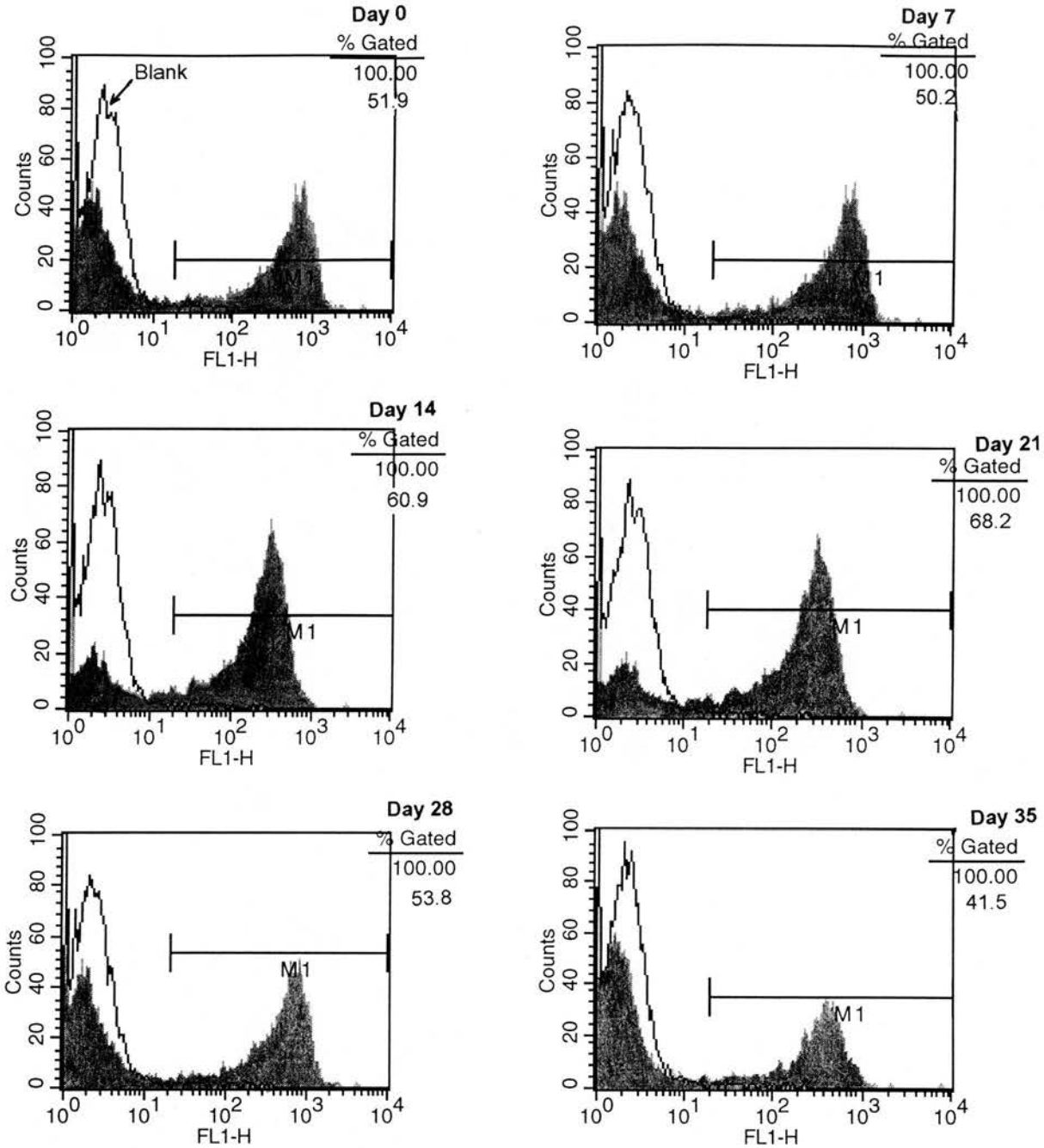


FIGURE C4: REPRESENTATIVE $CD5^+$ T CELL HISTOGRAMS FOR THE CONTROL GROUP(Sheep 1736)

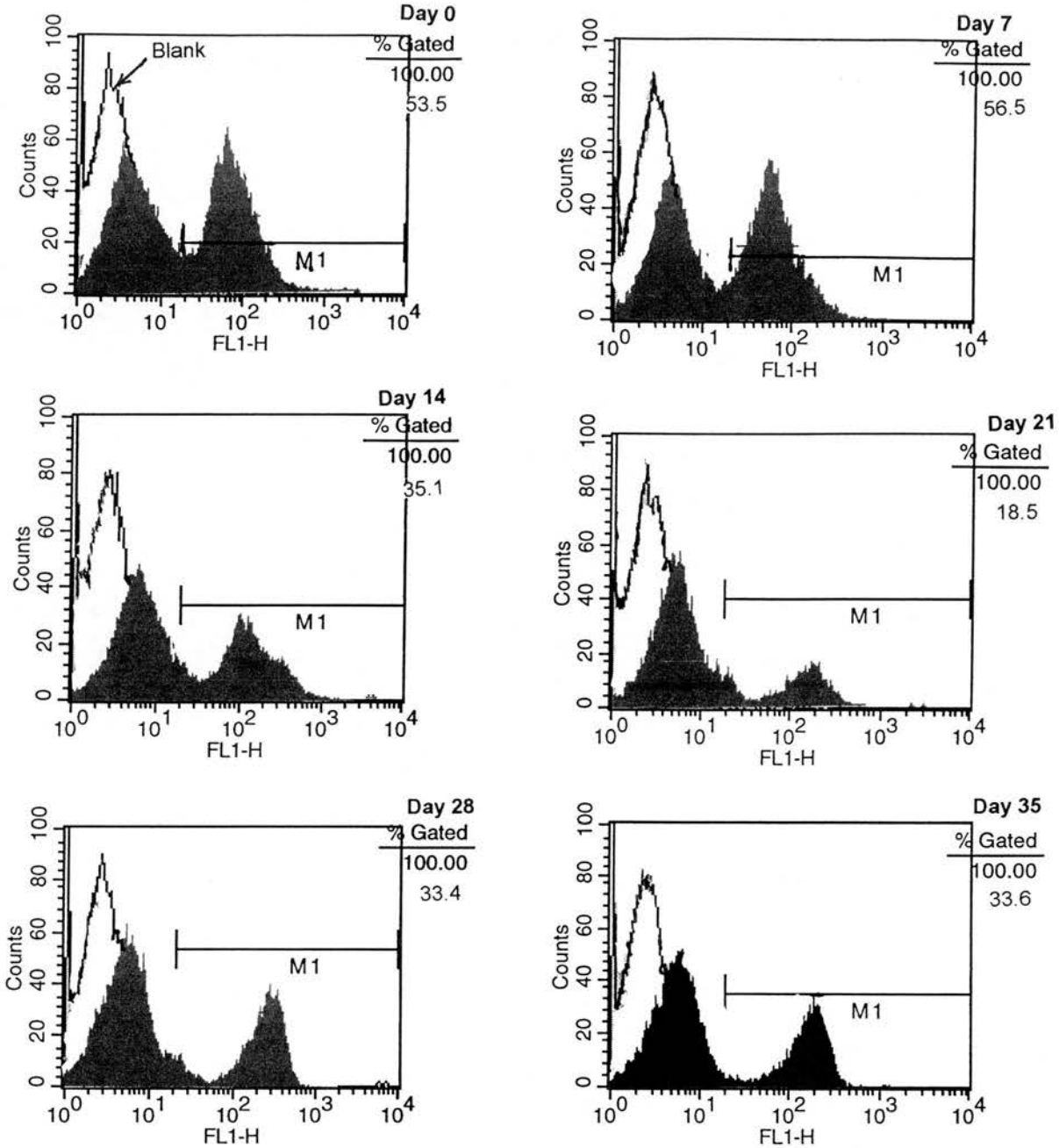


FIGURE C5: REPRESENTATIVE CD5$^+$ T CELL HISTOGRAMS FOR THE PROPHYLACTIC GROUP(Sheep 1826)

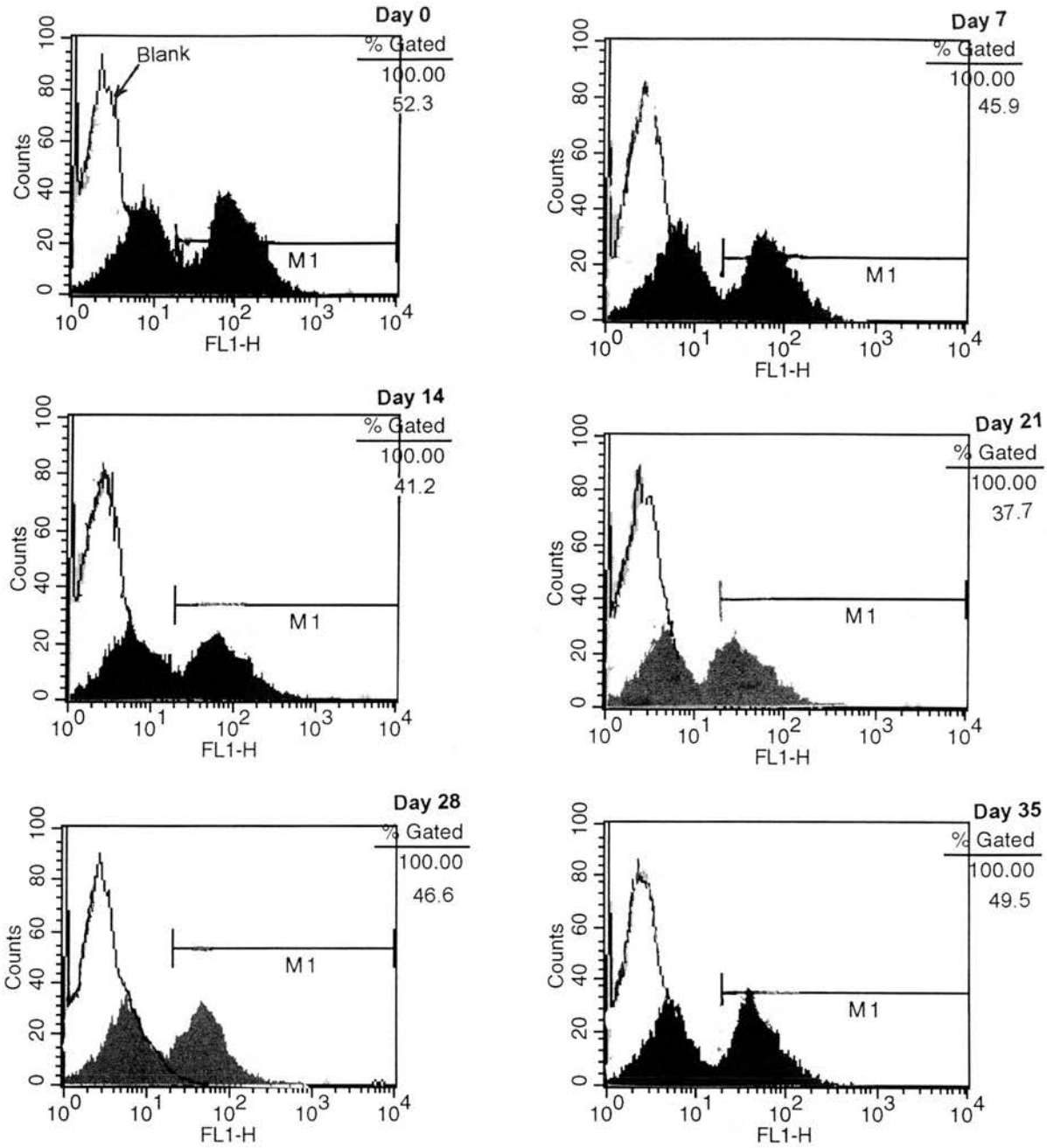


FIGURE C6: REPRESENTATIVE CD5$^+$ T CELL HISTOGRAMS FOR THE TREATED GROUP(Sheep 3WS)

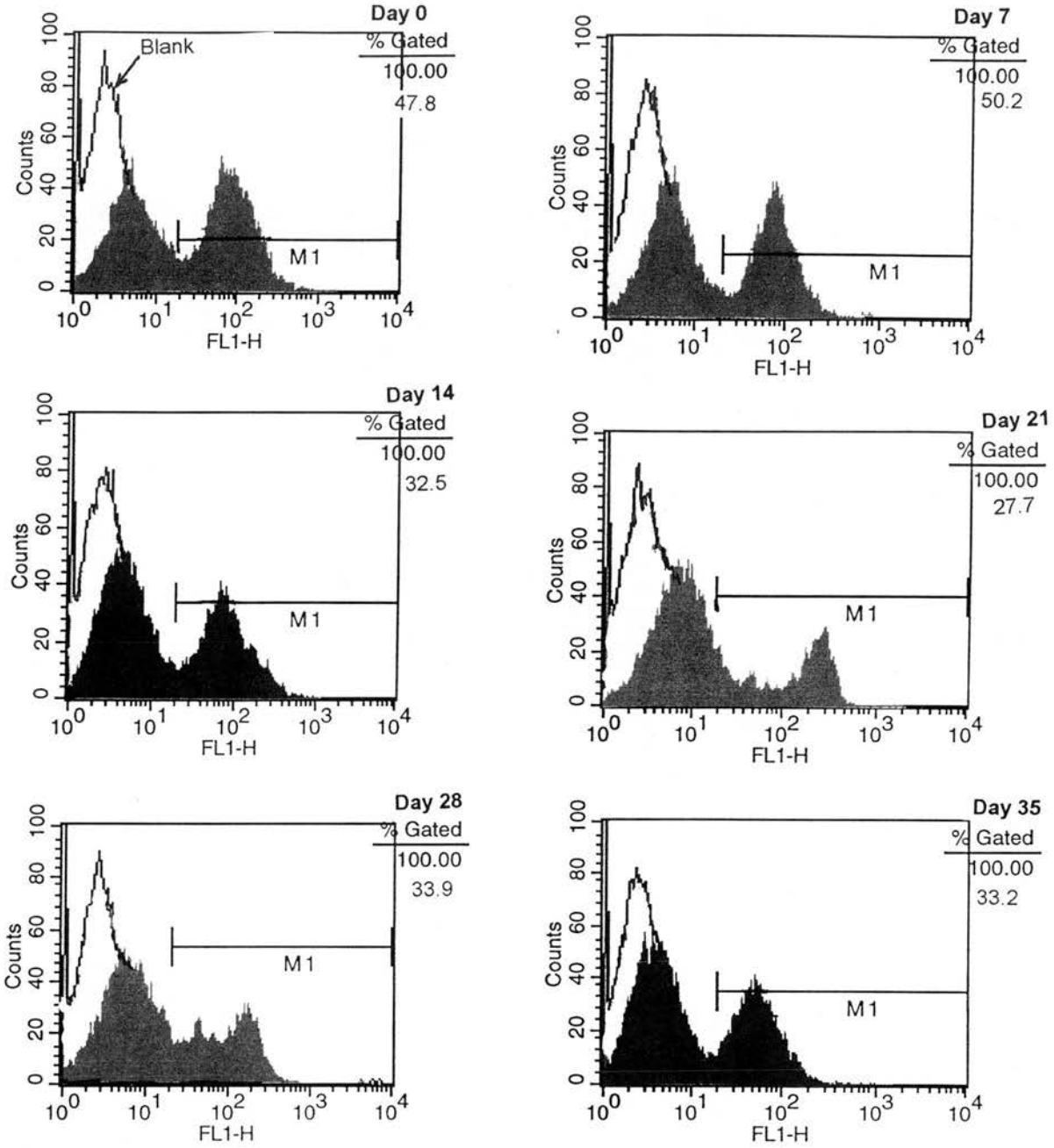


FIGURE C7: REPRESENTATIVE $\gamma\delta^+$ T CELL HISTOGRAMS FOR THE CONTROL GROUP(Sheep 1736)

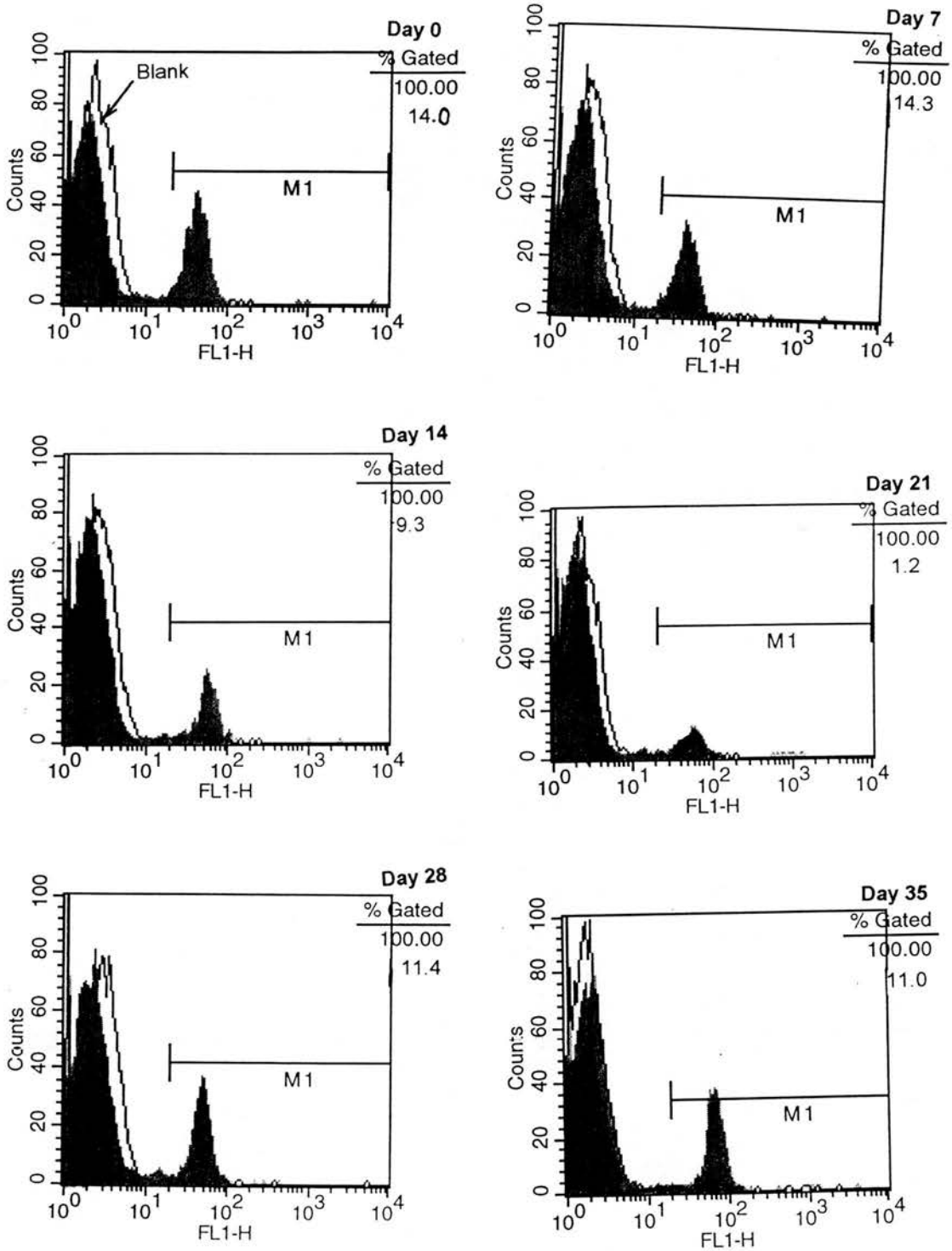


FIGURE C8: REPRESENTATIVE $\gamma\delta^+$ T CELL HISTOGRAMS FOR THE PROPHYLACTIC GROUP(Sheep 1826)

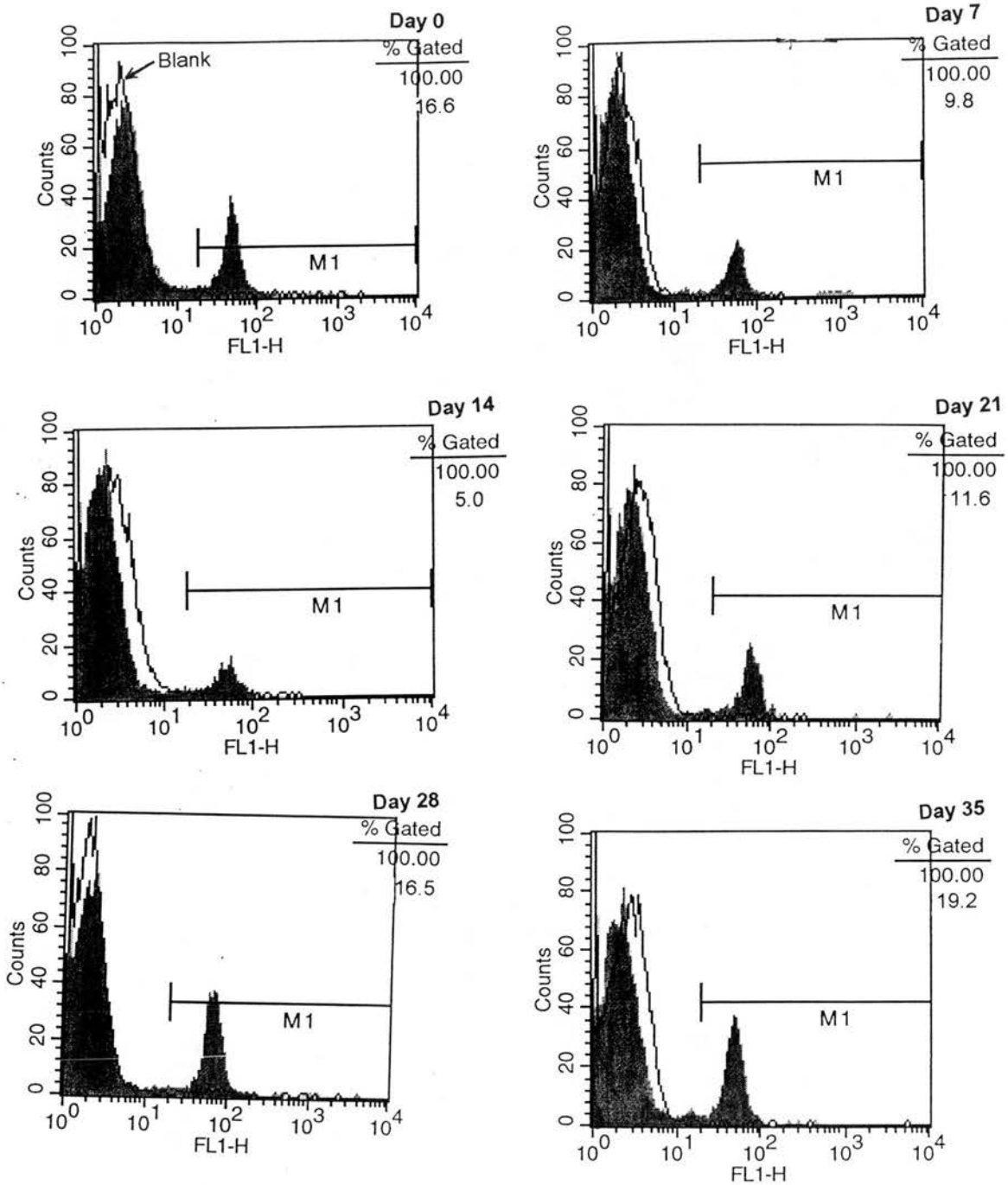


FIGURE C9: REPRESENTATIVE $\gamma\delta^+$ T CELL HISTOGRAMS FOR THE TREATED GROUP(Sheep 3WS)

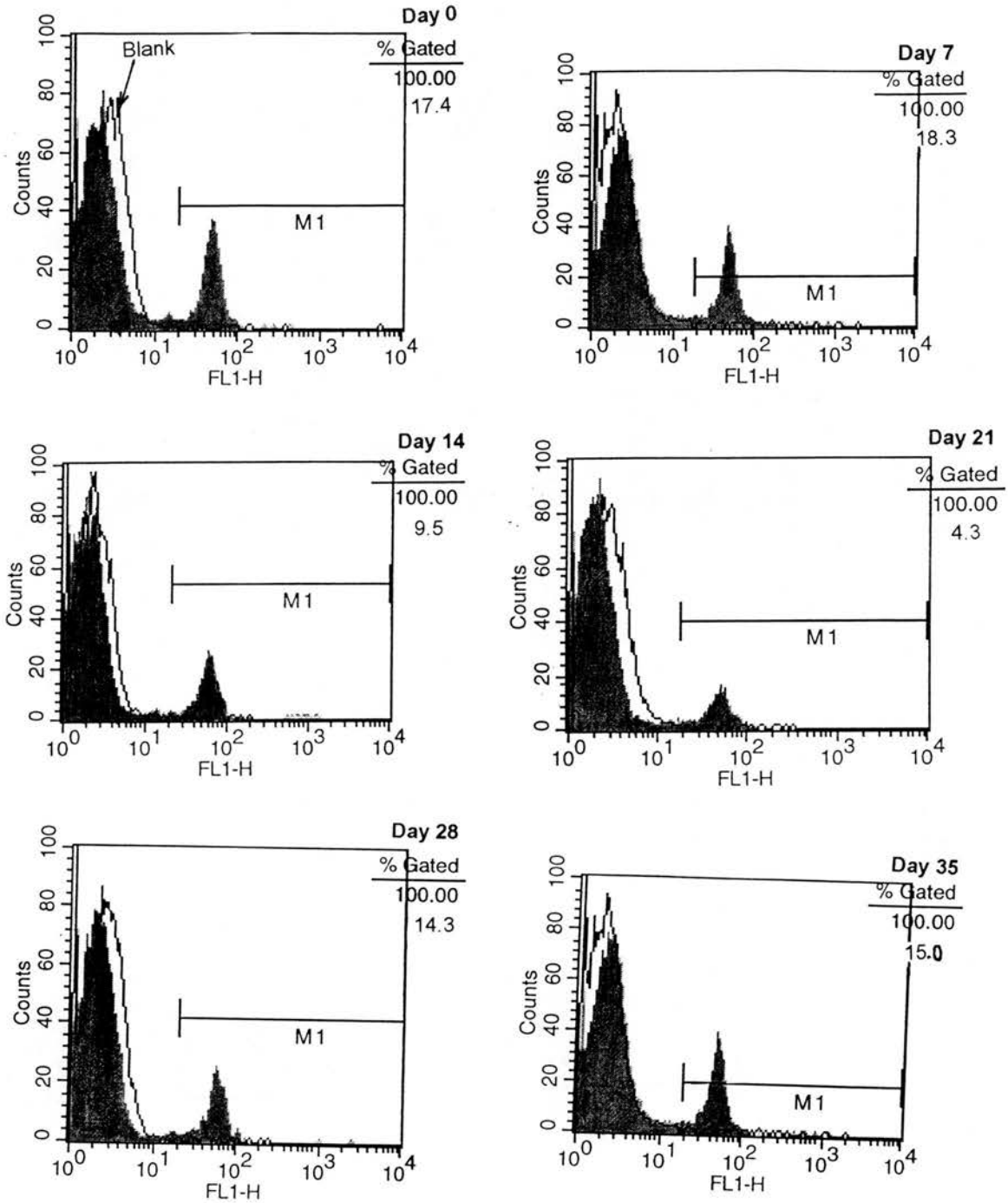


FIGURE C10: REPRESENTATIVE $CD8^+$ T CELL HISTOGRAMS FOR THE CONTROL GROUP(Sheep 1736)

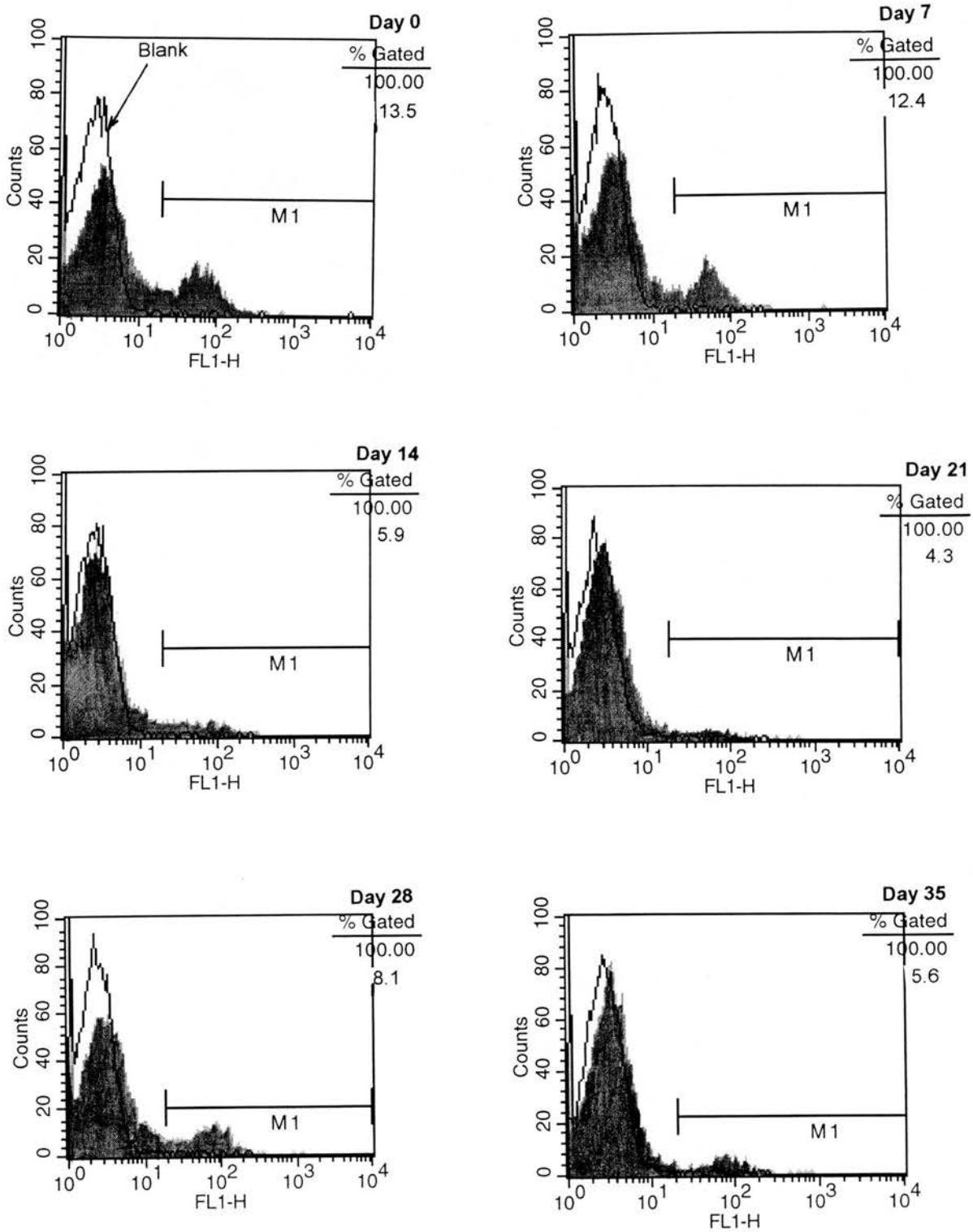


FIGURE C11: REPRESENTATIVE CD8$^+$ T CELL HISTOGRAMS FOR THE PROPHYLACTIC GROUP(Sheep 1826)

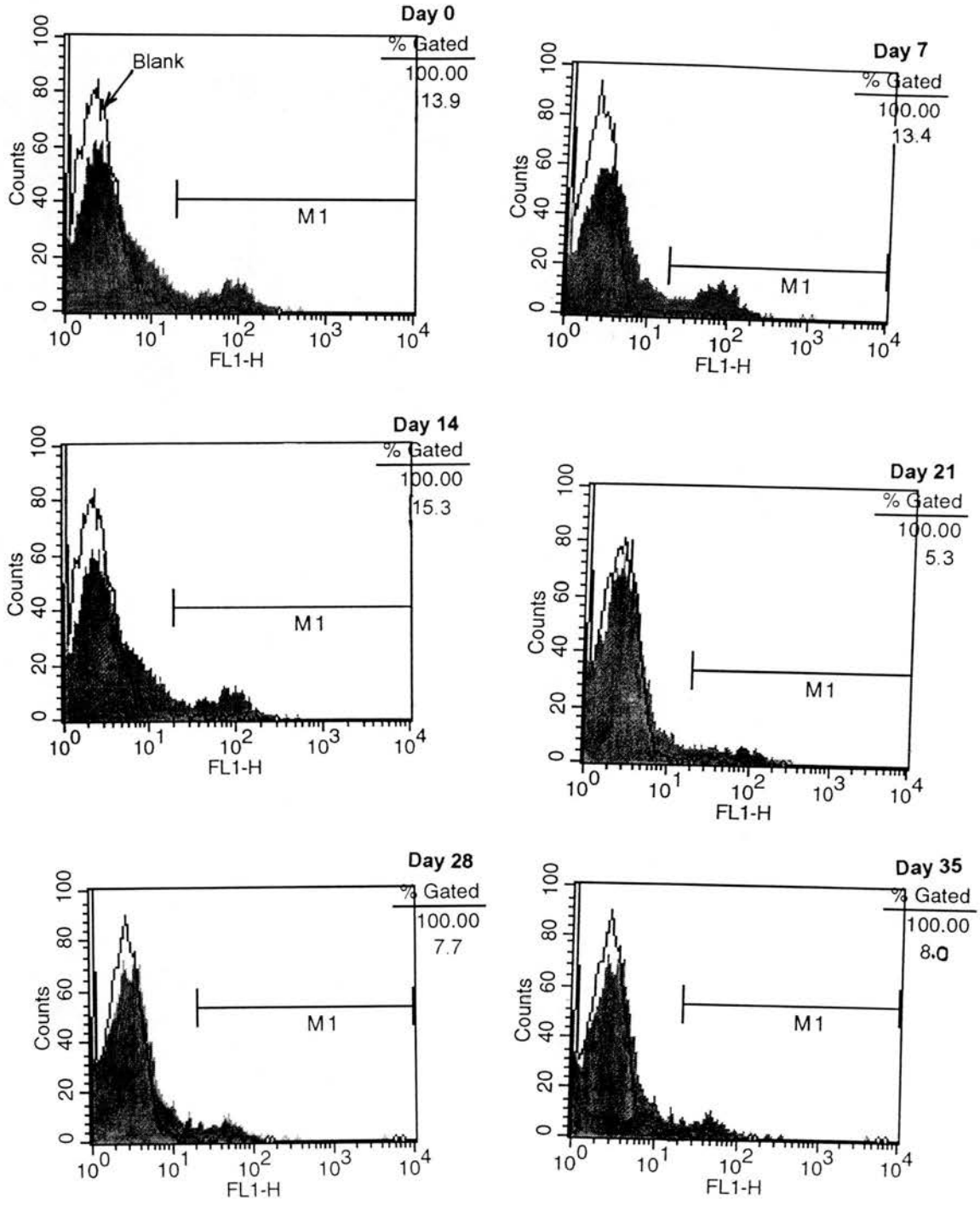


FIGURE C12: REPRESENTATIVE $CD8^+$ T CELL HISTOGRAMS FOR THE TREATED GROUP(Sheep 3WS)

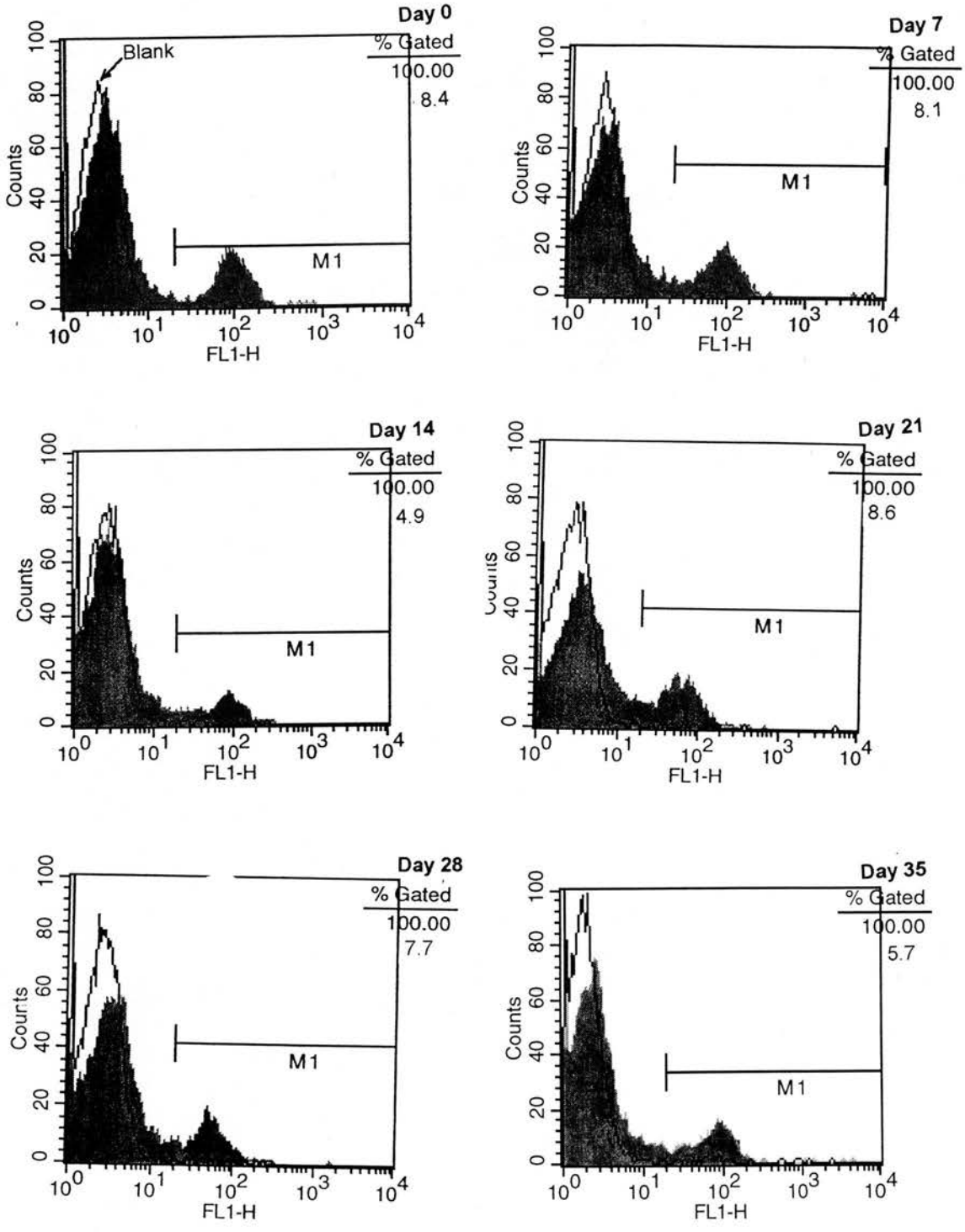


FIGURE C13: REPRESENTATIVE CD4$^+$ T CELL HISTOGRAMS FOR THE CONTROL GROUP(Sheep 1736)

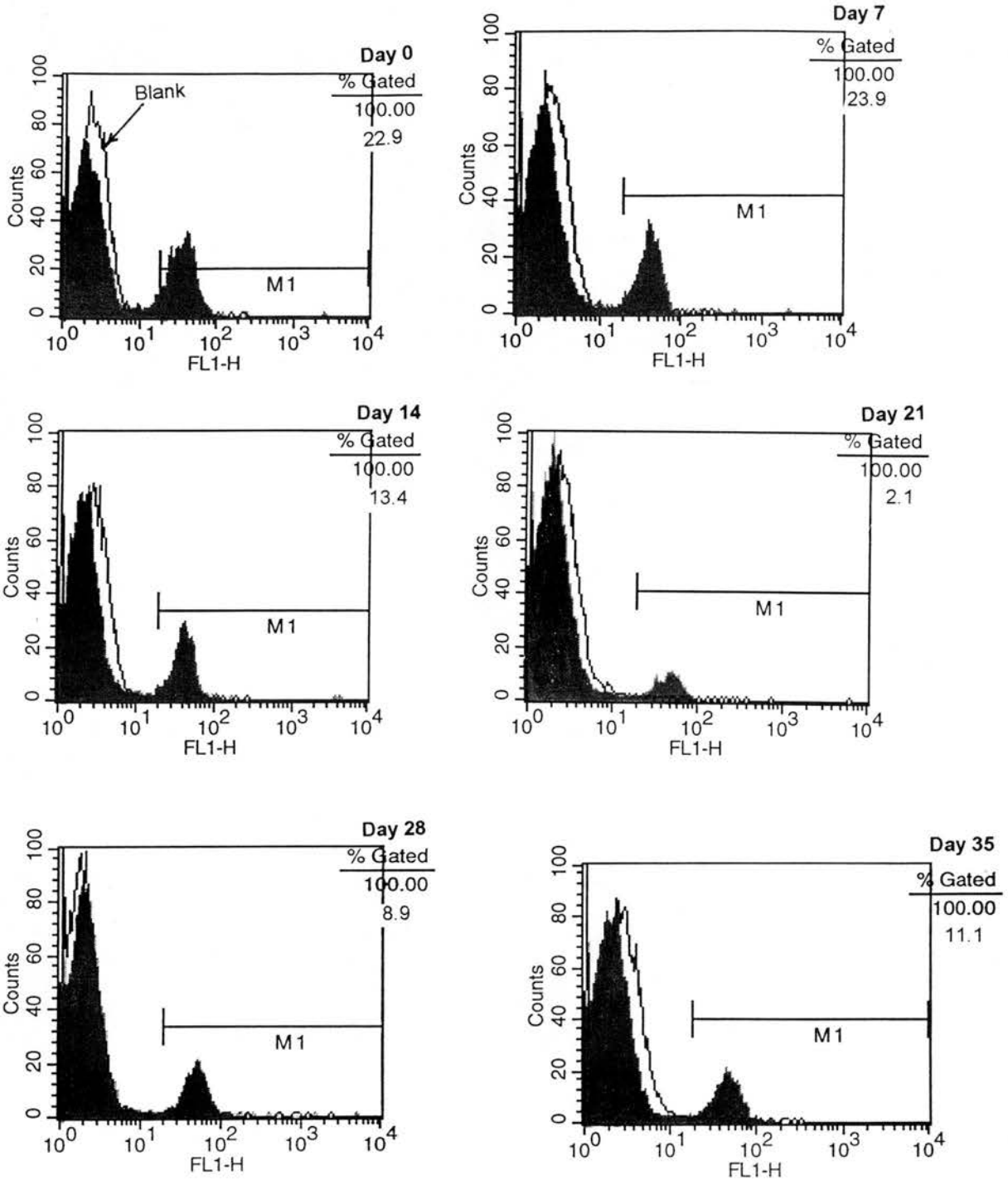


FIGURE C14: REPRESENTATIVE $CD4^+$ T CELL HISTOGRAMS FOR THE PROPHYLACTIC GROUP(Sheep 1826)

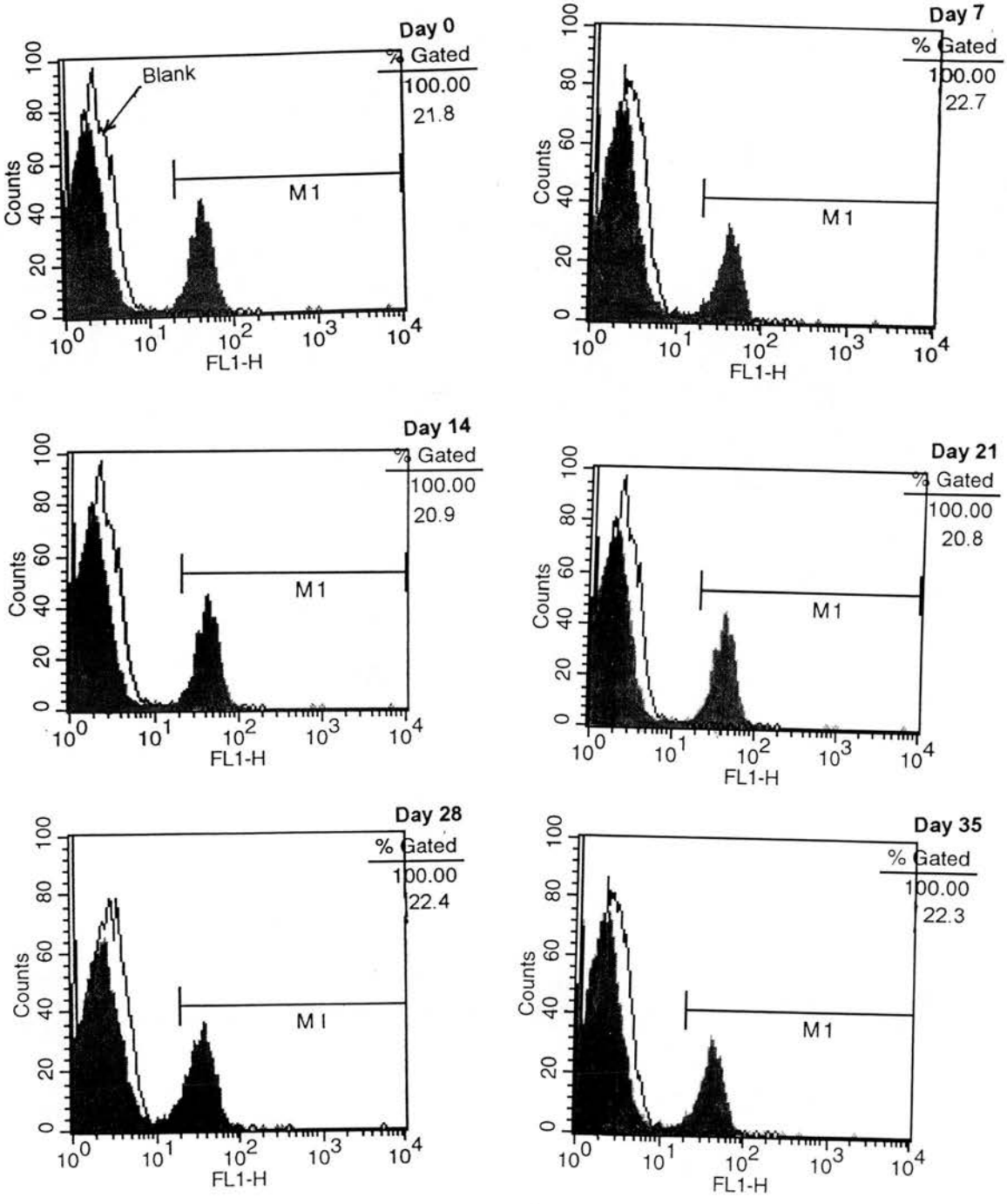
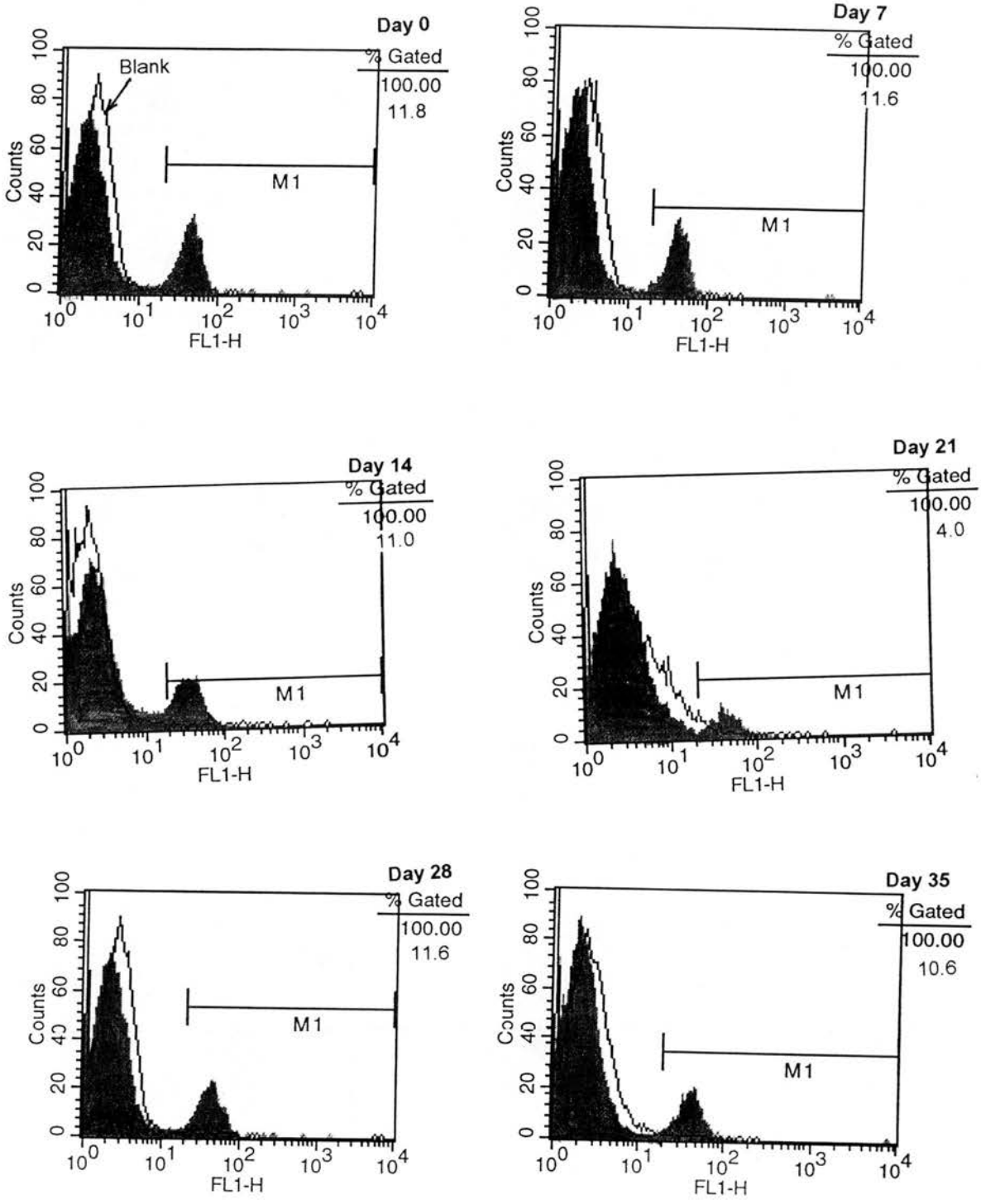


FIGURE C15: REPRESENTATIVE $CD4^+$ T CELL HISTOGRAMS FOR THE TREATED GROUP(Sheep 3WS)



APPENDIX V

TABLES OF RESULTS FOR IFN-γ, IL-12 AND PBMC *IN VITRO* PROLIFERATION

Table D1: Control group- Peripheral blood mononuclear cell proliferation (Stimulation Index) *in vitro*

Days pre/p	(a) Infected Control group - Tryps				(b) Infected Control group - ConA			
infection	1736	1863	2381	2383	1736	1863	2381	2383
-14	1.00	0.93	1.04	0.98	1.88	1.82	1.79	2.76
-7	0.95	1.01	0.98	1.03	1.95	1.78	1.68	2.92
0	0.93	0.95	0.92	0.94	1.83	2.03	1.95	2.89
7	1.03	1.20	0.83	1.24	1.94	1.88	1.56	2.90
14	0.99	1.00	0.90	1.02	1.19	0.86	1.07	1.51
21	0.13	0.02	0.00	1.34	0.13	0.02	0.00	1.34
28	0.88	0.92	1.06	0.80	1.44	0.75	0.67	1.27
35	1.67	1.64	1.38	1.68	1.30	1.18	0.69	1.30

Table D2: Prophylactic group- Peripheral blood mononuclear cell proliferation (Stimulation Index) *in vitro*

Days pre/p	(a) Infected Prophylactic group - Tryps				(b) Infected Prophylactic group - ConA			
infection	1618	1746	1826	1895	1618	1746	1826	1895
-14	1.37	1.30	1.39	1.37	3.04	1.97	2.91	2.77
-7	1.46	1.25	1.30	1.36	2.99	1.86	2.86	2.96
0	1.30	1.31	1.40	1.43	3.11	2.22	3.00	2.86
7	1.58	1.36	1.05	1.29	2.50	2.09	3.21	2.50
14	1.23	1.21	1.41	1.46	2.87	1.98	2.88	2.80
21	0.90	0.08	0.45	0.28	0.33	0.00	0.33	2.40
28	1.26	1.21	1.01	0.99	2.53	2.14	2.28	3.15
35	1.30	1.19	1.18	1.15	2.39	2.11	2.77	3.03

Table D3: Treated group- Peripheral blood mononuclear cell proliferation (Stimulation Index) *in vitro*

Days pre/p	(a) Infected Treated group - Tryps				(b) Infected Treated group - ConA			
infection	2BF	3WS	1499	2386	2BF	3WS	1499	2386
-14	0.95	1.01	0.99	1.03	1.06	1.73	1.83	2.94
-7	1.04	0.94	0.98	0.94	1.01	1.68	1.74	2.84
0	0.98	0.98	0.94	0.91	0 90	1.68	1.82	3.05
7	0.84	1.14	1.16	1.06	0.98	1.20	1.77	2.83
14	0.97	0.92	1.10	0.96	0.97	1.01	1.12	1.82
21	0.49	2.04	1.19	0.80	0.49	2.04	1.19	0.80
28	1.03	1.26	1.36	1.05	0.72	2.59	2.14	1.57
35	1.31	1.92	1.85	2.30	0.36	1.42	1.49	1.92

Multiplication Index = Mean of test absorbance/ Mean of media only absorbance.
It indicates the rate at which cells multiply. An index of 1 represents normal multiplication while that of zero indicate no cell multiplication. An index above 1 indicates a higher than normal rate of multiplication.

Table D4: Supernatant sheep IFN-γ levels (Absorbance) for prophylactic PBMCs in culture with trypanosomes

Days pre/p	(a). Non-infected (Prophylactic): Tryps				(b). Infected (Prophylactic): Tryps			
infection	1618	1746	1826	1895	1618	1746	1826	1895
-14	0.132	0.125	0.126	0.126	0.150	0.140	0.317	0.144
-7	0.074	0.045	0.020	0.048	0.185	0.092	0.298	0.153
0	0.160	0.142	0.130	0.150	0.122	0.138	0.329	0.168
7	0.110	0.113	0.120	0.198	0.177	0.125	0.325	0.180
14	0.574	0.288	0.498	0.713	0.158	0.128	0.300	0.130
21	0.162	0.207	1.230	0.572	0.252	0.140	0.306	0.460
28	0.181	0.122	0.876	0.265	0.172	0.113	0.168	0.093
35	0.180	0.145	0.424	0.141	0.160	0.077	0.176	0.104

Table D5: Supernatant sheep IFN-γ levels (Absorbance) for Control PBMCs in culture with trypanosomes

Days with	(a). Non-infected (Control): Tryps				(b). Infected (Control): Tryps			
to ISMM i	1736	1863	2381	2383	1736	1863	2381	2383
-14	0.143	0.149	0.159	0.150	0.147	0.136	0.134	0.151
-7	0.156	0.139	0.158	0.161	0.154	0.132	0.144	0.165
0	0.146	0.163	0.152	0.152	0.149	0.147	0.131	0.144
7	0.160	0.152	0.154	0.141	0.170	0.144	0.175	0.183
14	0.136	0.149	0.149	0.144	0.154	0.142	0.149	0.142
21	0.144	0.150	0.154	0.142	0.144	0.144	0.149	0.160
28	0.152	0.158	0.146	0.129	0.149	0.139	0.144	0.188
35	0.147	0.162	0.149	0.137	0.154	0.149	0.144	0.175

Table D6: Supernatant sheep IFN-γ levels (Absorbance) for Prophylactic PBMCs in culture with ConA

Days pre/p	(a). Non-infected (Prophylaxis): ConA				(b). Infected-Prophylactic group: ConA			
infection	1618	1746	1826	1895	1618	1746	1826	1895
-14	0.158	0.532	0.413	0.27	0.178	0.552	0.379	0.246
-7	0.132	0.517	0.404	0.228	0.136	0.564	0.419	0.222
0	0.17	0.54	0.432	0.23	0.160	0.528	0.400	0.239
7	0.139	0.546	0.408	0.23	0.132	0.536	0.410	0.262
14	0.186	0.543	0.395	0.256	0.170	0.554	0.408	0.225
21	0.176	0.542	0.394	0.241	0.146	0.562	0.432	0.284
28	0.178	0.51	0.418	0.221	0.166	0.550	0.390	0.256
35	0.141	0.52	0.394	0.216	0.142	0.594	0.395	0.220

Mean negative control = 0.139, SD = 0.012

Mean positive control = 1.259, SD = 0.05

Cut-off point = mean of negative control plus 2SD = 0.163

Values are means of duplicate sample tests performed twice

Prophylactic group: ISMM administered on day 0

Table D7: Supernatant sheep IFN-γ levels (Absorbance) for Control PBMCs in culture with ConA

Days pre/pc	(a). Non-infected (Control): ConA				(b). Infected (Control): ConA			
infection	1736	1863	2381	2383	1736	1863	2381	2383
-14	0.552	0.379	0.246	0.23	0.415	0.297	0.289	0.318
-7	0.564	0.419	0.222	0.198	0.386	0.304	0.304	0.324
0	0.528	0.4	0.239	0.21	0.441	0.387	0.355	0.269
7	0.536	0.41	0.262	0.224	0.345	0.252	0.278	0.283
14	0.554	0.408	0.225	0.215	0.412	0.242	0.309	0.306
21	0.562	0.432	0.284	0.193	0.432	0.337	0.276	0.234
28	0.55	0.39	0.256	0.233	0.342	0.257	0.219	0.340
35	0.594	0.395	0.22	0.246	0.345	0.325	0.281	0.312

Table D8: Non-infected splenic mouse IL-12 and IFN-γ levels (Absorbance)

	IL-12 production (Absorbance)				IFNγ production (Absorbance)			
	Days post ISMM adminstration				Days post ISMM adminstration			
	0	7	14	21	0	7	14	21
(a) Media only								
Mouse 1	0.011	0.004	0.009	0.011	0.014	0.009	0.011	0.016
Mouse 1	0.009	0.008	0.011	0.010	0.015	0.011	0.015	0.013
Mouse 2	0.012	0.005	0.010	0.011	0.012	0.008	0.006	0.011
Mouse 2	0.011	0.006	0.012	0.013	0.013	0.010	0.017	0.009
Mouse 3	0.012	0.005	0.010	0.012	0.010	0.007	0.012	0.010
Mouse 3	0.010	0.004	0.009	0.011	0.011	0.009	0.013	0.012
Median	**0.011**	**0.005**	**0.010**	**0.011**	**0.013**	**0.009**	**0.013**	**0.012**
(b) LPS culture								
Mouse 1	0.141	0.163	0.041	0.037	0.393	0.010	0.013	0.009
Mouse 1	0.140	0.172	0.038	0.043	0.401	0.007	0.011	0.010
Mouse 2	0.142	0.140	0.023	0.065	0.389	0.009	0.009	0.012
Mouse 2	0.143	0.155	0.034	0.058	0.421	0.015	0.015	0.015
Mouse 3	0.167	0.134	0.035	0.048	0.354	0.006	0.012	0.011
Mouse 3	0.150	0.146	0.033	0.050	0.335	0.017	0.013	0.009
Median	**0.143**	**0.151**	**0.035**	**0.049**	**0.391**	**0.010**	**0.013**	**0.011**
(c) Trypanosome culture								
Mouse 1	0.159	0.232	0.243	0.018	0.023	0.005	0.018	0.015
Mouse 1	0.192	0.205	0.268	0.020	0.018	0.006	0.022	0.012
Mouse 2	0.183	0.227	0.231	0.024	0.022	0.005	0.017	0.013
Mouse 2	0.230	0.247	0.276	0.034	0.017	0.004	0.023	0.014
Mouse 3	0.213	0.231	0.251	0.019	0.016	0.010	0.016	0.011
Mouse 3	0.224	0.201	0.212	0.021	0.019	0.007	0.018	0.010
Median	**0.203**	**0.229**	**0.247**	**0.021**	**0.019**	**0.006**	**0.018**	**0.013**

APPENDIX VI
DATA FOR BCG EXPERIMENTS

Table E1 *In vivo* **PBMC proliferation (% Lymphoblasts) following BCG inoculation**

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	16	21	24	12	21	20
-7	15	20	25	10	19	22
0	14	18	21	12	18	21
7	17	21	23	10	19	23
14	28	22	27	11	23	24
21	31	33	33	24	27	24
28	31	38	44	19	16	19
35	34	24	22	18	31	15

Table E2 Percentage of B-cell following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	28.0	48.8	46.0	39.2	44.8	39.2
-7	26.7	48.6	46.4	39.4	42.3	37.5
0	28.8	52.7	50.2	40.2	44.8	46.0
7	20.4	43.3	40.3	34.2	45.2	28.3
14	20.3	38.8	36.6	32.0	31.9	26.3
21	15.7	29.3	25.5	29.0	21.2	14.7
28	20.6	76.4	68.7	29.6	22.7	41.5
35	62.4	77.1	72.5	30.1	24.6	47.7

Table E3 Percentage of $CD5^+$ T-cells following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	70.2	49.6	46.2	40.5	45.9	55.7
-7	68.4	46.3	44.1	41.8	46.0	52.4
0	66.0	45.7	39.8	43.4	50.3	50.1
7	69.8	51.0	42.7	46.4	44.8	58.1
14	70.4	50.4	44.4	48.5	53.1	62.6
21	70.7	41.9	37.6	36.2	46.3	55.0
28	28.3	14.4	23.8	46.2	68.6	48.5
35	26.2	37.4	24.1	44.6	63.7	32.9

Table E4 Percentage of $\gamma\delta^+$ T-cells following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	33.5	27.4	22.6	25.6	21.6	22.9
-7	30.5	26.0	19.8	23.4	19.6	22.7
0	31.4	25.5	21.4	26.1	17.3	21.3
7	31.2	25.6	17.7	30.1	19.9	20.4
14	36.3	26.0	17.2	29.2	20.7	18.5
21	14.1	10.4	3.4	12.2	8.3	5.0
28	11.6	3.2	7.8	31.3	33.9	19.4
35	12.4	3.6	9.3	35.2	42.8	21.3

Table E5 Percentage of $CD4^+$ T-cells following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	23.0	11.2	14.6	11.1	15.0	19.0
-7	22.4	10.6	14.9	12.3	16.3	16.8
0	20.5	10.8	15.7	14.0	17.3	16.6
7	24.3	13.9	15.6	11.4	17.0	21.2
14	23.1	12.2	14.5	13.5	16.0	23.2
21	26.7	11.4	12.9	10.6	16.4	21.3
28	5.2	3.1	6.0	7.1	20.0	11.1
35	6.3	4.2	4.1	9.6	19.6	12.6

Table E6 Percentage of $CD8^+$ T-cells following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	12.9	8.8	7.5	4.2	14.3	15.9
-7	12.1	8.9	8.6	4.0	14.8	14.4
0	13.2	9.4	9.4	4.3	15.4	13.7
7	12.5	5.2	6.5	3.0	11.9	15.4
14	7.5	6.3	8.0	2.5	9.8	14.8
21	6.9	4.8	4.9	2.0	6.9	12.9
28	4.8	2.2	5.8	0.9	9.3	13.6
35	6.2	4.2	5.1	1.2	8.7	7.4

Table E7 $CD4^+$: $CD8^+$ T-cell ratio following BCG inoculation

Days pre/post inoculation	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
-14	1.78	1.27	1.95	2.64	1.05	1.19
-7	1.85	1.19	1.73	3.08	1.10	1.17
0	1.55	1.15	1.67	3.26	1.12	1.21
7	1.94	2.67	2.40	3.80	1.43	1.38
14	3.08	1.94	1.80	5.40	1.63	1.57
21	3.87	2.38	2.63	5.30	2.38	1.65
28	1.08	1.41	1.03	7.89	2.15	1.77
35	1.02	1.00	0.80	8.00	2.25	1.70

Table E8 Skin thickness (mm) after PPD injection 35 days post BCG inoculation

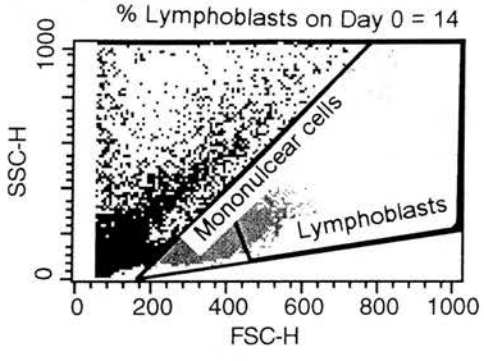
Hours post PPD injection	(a) CONTROL GROUP			(b) ISMM GROUP		
	2212	2305	NT	2266	2327	2378
0	5.8	5.8	5.5	5.6	5.7	4.7
24	11.6	11.1	8.9	13.8	14.6	5.0
48	15.4	21.3	9.8	18.4	18.5	5.0
72	13.3	22.4	10.0	19.2	19.6	7.0

~ BCG inoculation was performed on Day 0

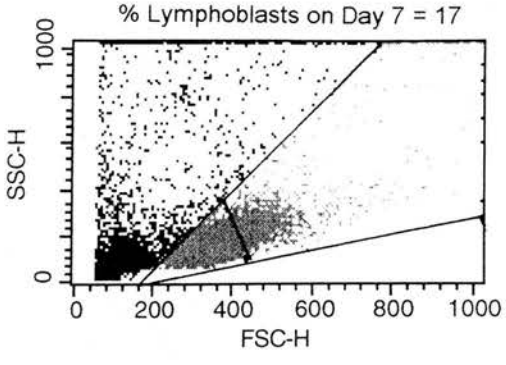
~ ISMM administered 14 days before BCG inoculation

FIGURE E1: SHEEP 2212- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

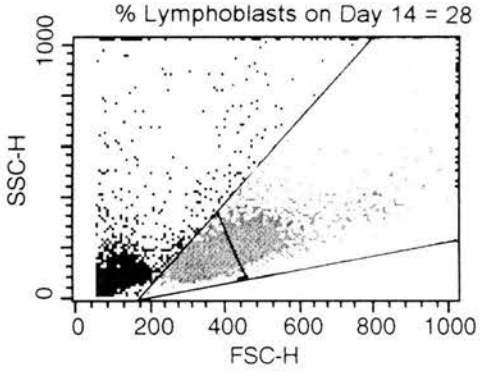
Region	Events
ALL LYMPH	9872
BLASTS	1382



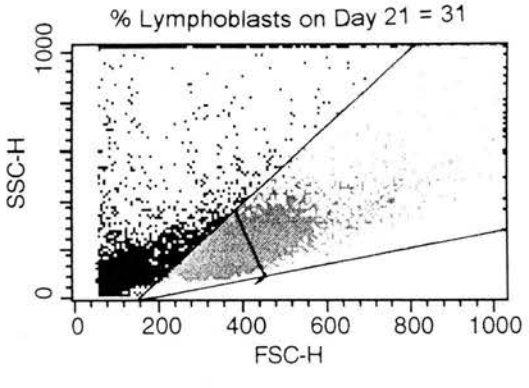
Region	Events
ALL LYMPH	10177
BLASTS	1730



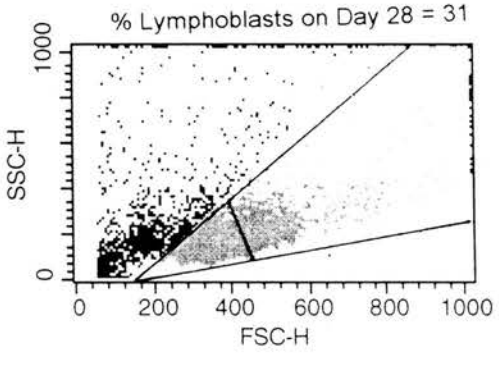
Region	Events
ALL LYMPH	9923
BLASTS	2733



Region	Events
ALL LYMPH	9789
BLASTS	3021



Region	Events
ALL LYMPH	9882
BLASTS	3021



Region	Events
ALL LYMPH	9405
BLASTS	3219

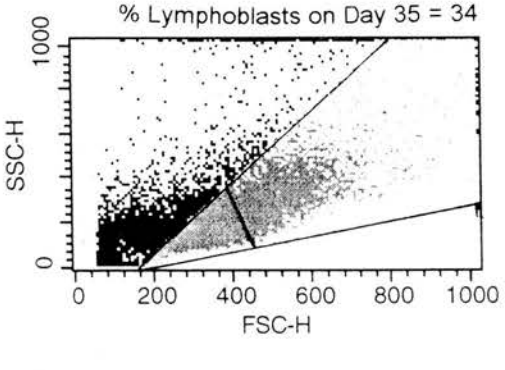
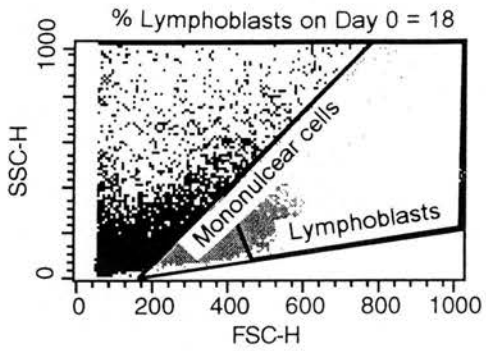
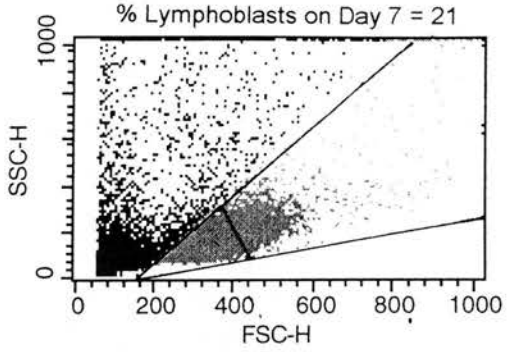


FIGURE E2: SHEEP 2305- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

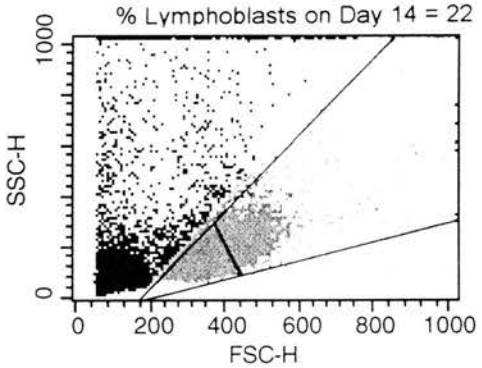
Region	Events
ALL LYMPH	9599
BLASTS	1728



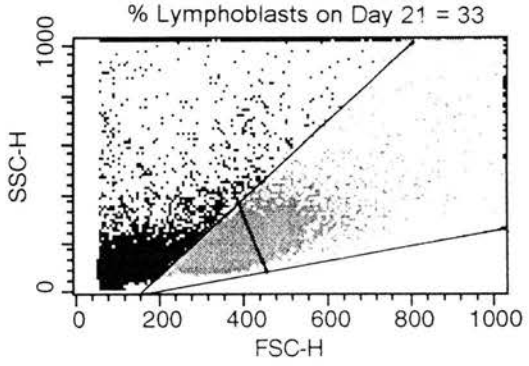
Region	Events
ALL LYMPH	10111
BLASTS	2123



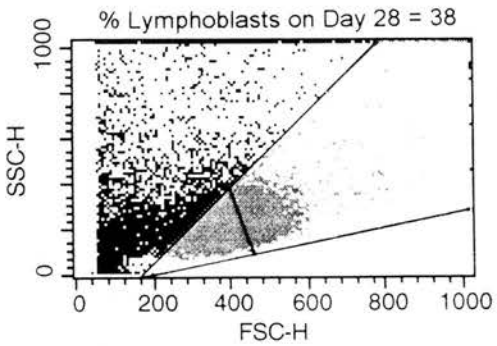
Region	Events
ALL LYMPH	9623
BLASTS	2090



Region	Events
ALL LYMPH	9576
BLASTS	3166



Region	Events
ALL LYMPH	9621
BLASTS	3698



Region	Events
ALL LYMPH	9582
BLASTS	2256

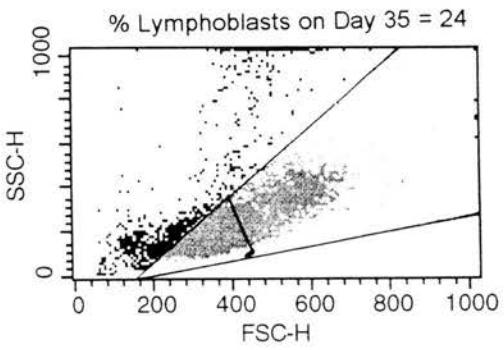
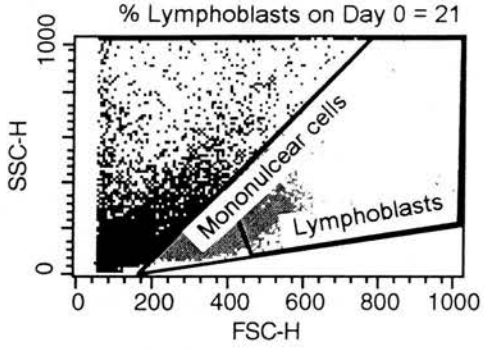
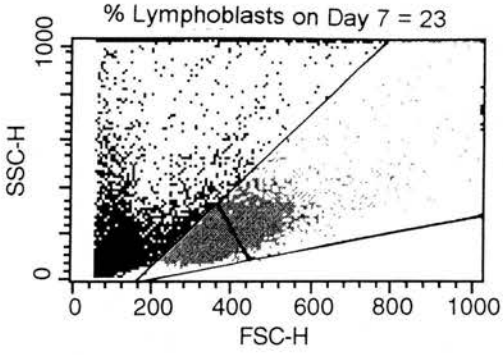


FIGURE E3: SHEEP NT- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

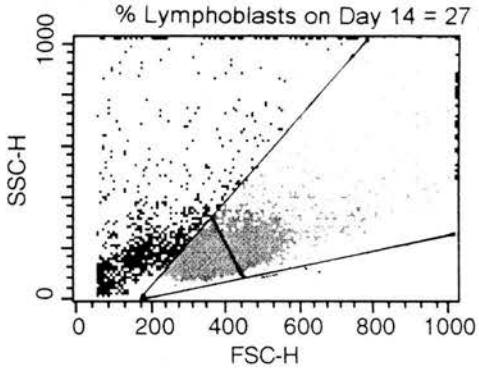
Region	Events
ALL LYMPH	9679
BLASTS	2032



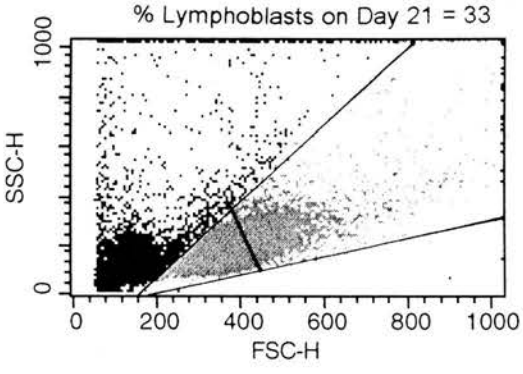
Region	Events
ALL LYMPH	10135
BLASTS	2331



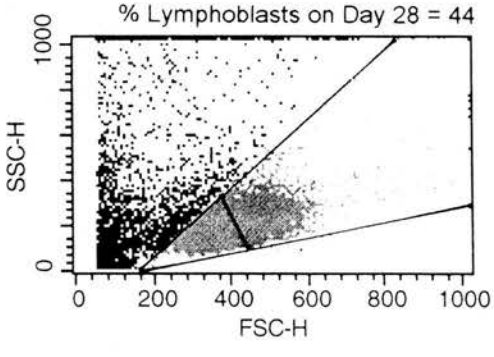
Region	Events
ALL LYMPH	9882
BLASTS	2668



Region	Events
ALL LYMPH	9225
BLASTS	3036



Region	Events
ALL LYMPH	9822
BLASTS	4340



Region	Events
ALL LYMPH	9623
BLASTS	2090

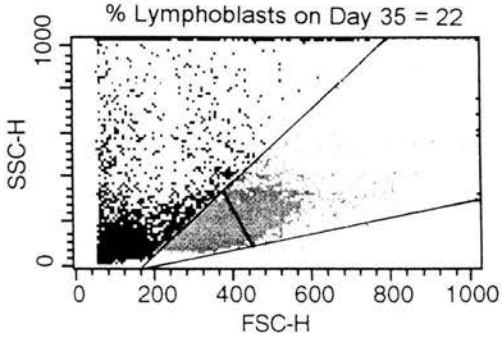
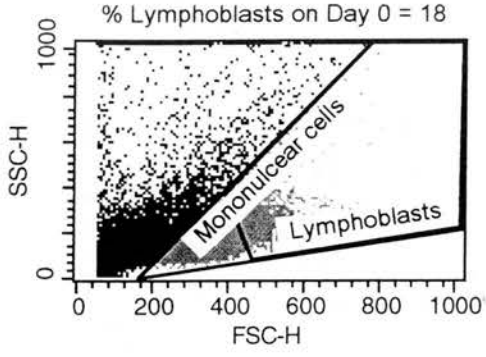
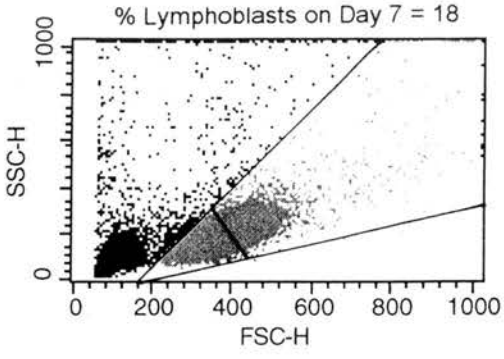


FIGURE E4: SHEEP 2327- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

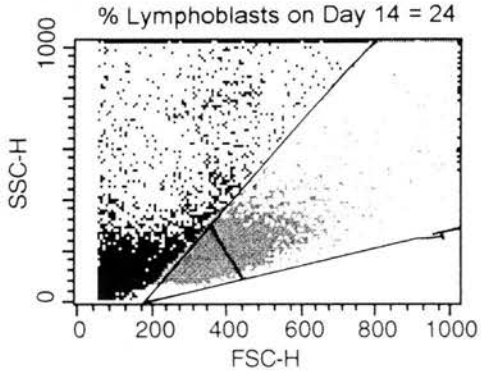
Region	Events
ALL LYMPH	9135
BLASTS	1644



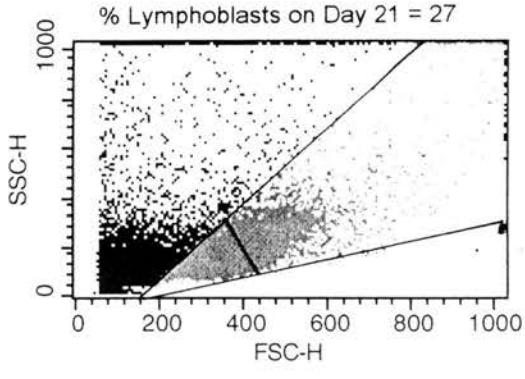
Region	Events
ALL LYMPH	10200
BLASTS	1819



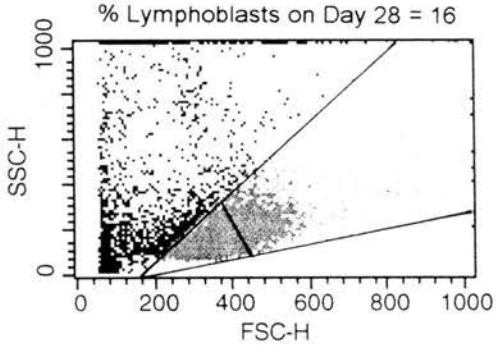
Region	Events
ALL LYMPH	9775
BLASTS	2348



Region	Events
ALL LYMPH	9445
BLASTS	2555



Region	Events
ALL LYMPH	9815
BLASTS	1557



Region	Events
ALL LYMPH	5557
BLASTS	1701

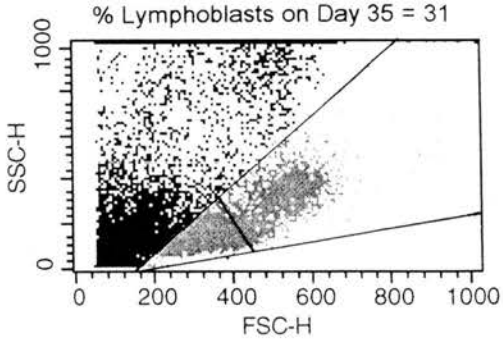
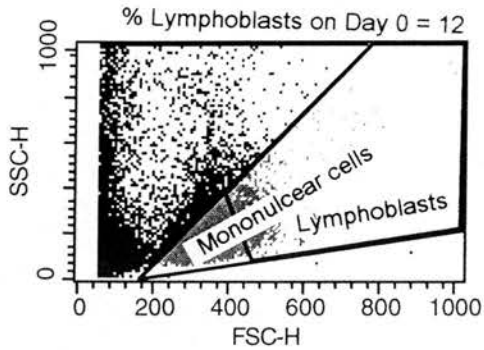
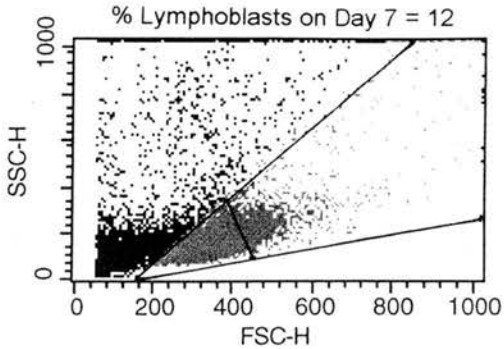


FIGURE E5: SHEEP 2266- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

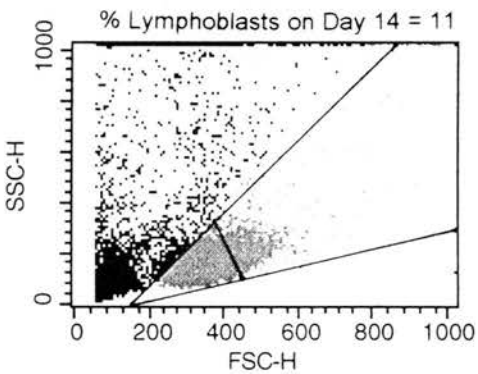
Region	Events
ALL LYMPH	9574
BLASTS	1185



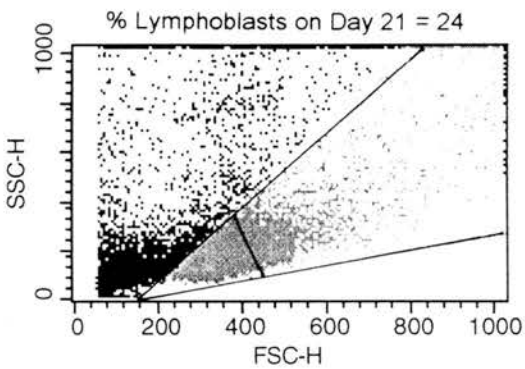
Region	Events
ALL LYMPH	9664
BLASTS	1203



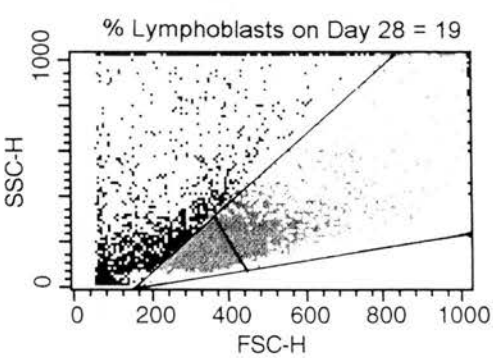
Region	Events
ALL LYMPH	9833
BLASTS	1056



Region	Events
ALL LYMPH	9775
BLASTS	2348



Region	Events
ALL LYMPH	9852
BLASTS	1893



Region	Events
ALL LYMPH	10200
BLASTS	1819

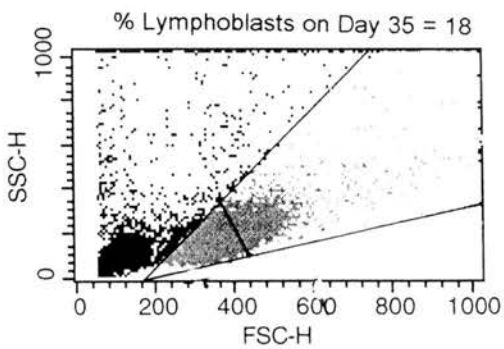
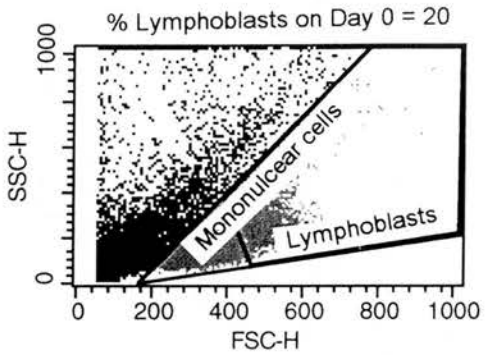
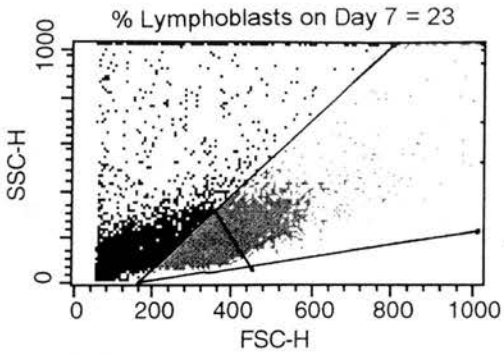


FIGURE E6: SHEEP 2378- DOT PLOTS FOR DETERMINING LYMPHOCYTE PROLIFERATION *IN VIVO* AFTER BCG INOCULATION

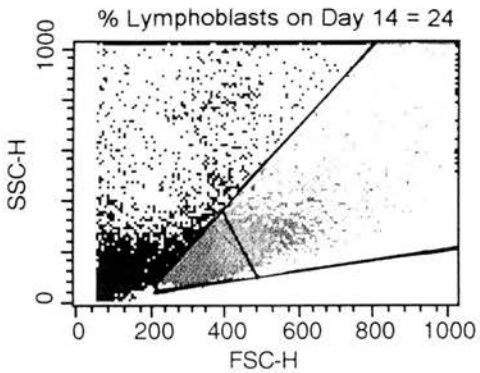
Region	Events
ALL LYMPH	9484
BLASTS	1907



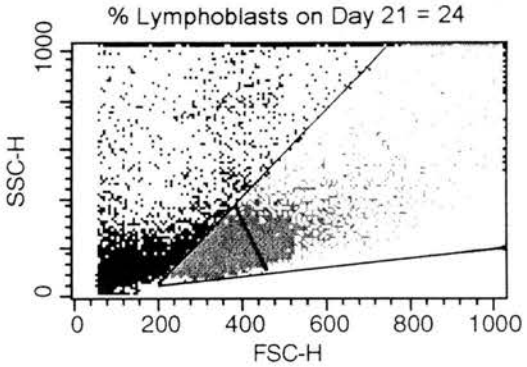
Region	Events
ALL LYMPH	9662
BLASTS	2222



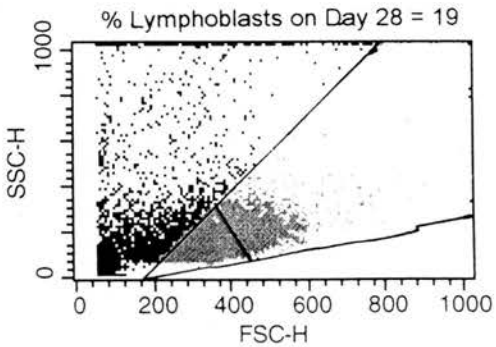
Region	Events
ALL LYMPH	9775
BLASTS	2348



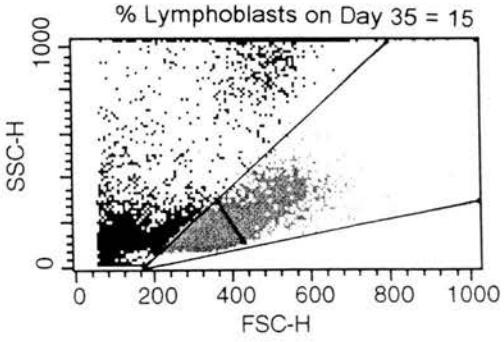
Region	Events
ALL LYMPH	9775
BLASTS	2348



Region	Events
ALL LYMPH	9696
BLASTS	1876



Region	Events
ALL LYMPH	9370
BLASTS	1400



APPENDIX VII
STATISTICAL ANALYSIS OF RESULTS
(Mann-Whiteny Non-parametric tests)

F1. STATISTICAL ANALYSIS OF FEVER AND HAEMATOLOGICAL RESULTS

Table F1.1 MAGINITUDE OF FEVER

COMPARISONS	Median (mm^2)	P-value	Comment
(a) Control & prophylactic Groups			
Control (0-35 dpi)	4825		
Prophylactic (0-35dpi)	150	0.029	Significant difference
(b) Control & treated Groups			
Control (0-35 dpi)	4825		
Treated (0-35 dpi)	2775	0.029	Significant difference
(c) Prophylactic & Treated Groups			
Prophylactic (0-35dpi)	150		
Treated (0-35 dpi)	2775	0.029	Significant difference

Table F1.2 PCV

COMPARISONS	Median (%)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	32		
14 ~ 35 days	28	<0.0001	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	38		
14 ~ 35 days	38	0.8900	Not significant
(c) Treated Group			
-14 ~ 0 days	35		
14 ~ 28 days	31	<0.0001	Significant decrease

Table F1.2 Absolute RBCs

COMPARISONS	Median ($x10^9$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	11.5		
14 ~ 35 days	10.0	0.007	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	12.4		
14 ~ 35 days	12.0	0.341	Not significant
(c) Treated			
-14 ~ 0 days	12.6		
14 ~ 35 days	12.6	0.659	Not significant

Table F1.3 Absolute WBCs

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	7.2		
14 ~ 35 days	11.2	0.0001	Significant increase
(b) Prophylactic Group			
-14 ~ 0 days	7.8		
14 ~ 35 days	8.8	0.472	Not significant
(c) Treated Group			
-14 ~ 0 days	10.0		
14 ~ 35 days	11.5	0.057	Not significant

Table F1.4 Absolute Lymphocytes

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	4.8		
14 ~ 35 days	9.3	<0.0001	Significant increase
(b) Prophylactic Group			
-14 ~ 0 days	5.7		
14 ~ 35 days	6.5	0.626	Not significant
(c) Treated Group			
-14 ~ 0 days	5.6		
14 ~ 28 days	8.9	0.002	Significant increase

Table F1.5 Absolute Monocytes

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.23		
14 ~ 35 days	0.42	0.022	Significant increase
(b) Prophylactic Group			
-14 ~ 0 days	0.31		
14 ~ 35 days	0.34	0.853	Not significant
(c) Treated Group			
-14 ~ 0 days	0.44		
14 ~ 21 days	0.83	0.004	Significant increase

Table F1.6 Absolute Neutrophils

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	1.8		
14 ~ 35 days	1.7	0.594	Not significant
(b) Prophylactic Group			
-14 ~ 0 days	1.5		
14 ~ 35 days	1.6	0.763	Not significant
(c) Treated Group			
-14 ~ 0 days	2.8		
14 ~ 28 days	2.4	0.256	Not significant

Table F1.7 Absolute Eosinophils

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.22		
14 ~ 35 days	0.20	0.194	Not significant
(b) Prophylactic Group			
-14 ~ 0 days	0.26		
14 ~ 35 days	0.26	0.710	Not significant
(c) Treated Group			
-14 ~ 0 days	0.24		
14 ~ 28 days	0.16	0.082	Not significant

F2. STATISTICAL ANALYSIS FOR EFFICACY OF ISMM IN MICE

Table F2.1 Prepatent period

COMPARISONS	Median (days)	P-value	Comment
(a) Control & ISMM Groups			
Control	3		
ISMM	7	0.008	Significant difference
(b) Control & ISMM+Hydro. Groups			
Control	3		
ISMM+Hydro.	3	0.841	Not significant
(c) ISMM & ISMM+Hydro. Groups			
ISMM	7		
ISMM+Hydro.	3	0.008	Significant difference

Table F2.2 Survival time

COMPARISONS	Median (days)	P-value	Comment
(a) Control & ISMM Groups			
Control	18		
ISMM	26	0.016	Significant difference
(b) Control & ISMM+Hydro. Groups			
Control	18		
ISMM+Hydro.	17	1.000	Not significant
(c) ISMM & ISMM+Hydro. Groups			
ISMM	26		
ISMM+Hydro.	17	0.095	Not significant

F3. STATISTICAL ANALYSIS FOR CELL PHENOTYPES AND IgG ANTIBODIES

Table F3.1 Absolute B cells

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	2.3		
14 ~ 28 days	5.3	<0.0001	Significant increase
(b) Prophylactic Group			
-14 ~ 0 days	2.6		
14 ~ 28 days	3.2	0.1570	Not significant
(c) Treated Group			
-14 ~ 0 days	2.2		
14 ~ 28 days	5.3	0.0004	Significant increase

Table F3.2 Absolute $CD5^+$ T cells

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	2.2		
7~ 14 days	2.9	0.003	Significant increase
(b) Prophylactic Group			
-14 ~ 0 days	2.7		
14 ~ 35 days	2.7	0.885	Not significant
(c) Treated Group			
-14 ~ 0 days	2.7		
14 ~ 35 days	2.7	0.816	Not significant

Table F3.3 Absolute $\gamma\delta^+$ T cells

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.76		
21 days	0.33	0.110	Not significant
(b) Prophylactic Group			
-14 ~ 0 days	0.95		
21 days	0.42	0.131	Not significant
(c) Treated Group			
-14 ~ 0 days	1.20		
14 ~ 21 days	0.53	0.038	Significant decrease

Table F3.4 Absolute $CD8^+$ T cells

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.50		
7~ 35 days	0.38	0.781	Not significant
(b) Prophylactic Group(Sheep 1618 & 1826)			
-14 ~ 0 days	0.76		
21 ~ 35 days	0.47	0.026	Significant decrease
(c) Treated Group			
-14 ~ 0 days	0.58		
21~ 35 days	0.80	0.003	Significant increase

Table F3.5 Absolute $CD4^+$ T cells

COMPARISONS	Median ($x10^6$/ml)	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.95		
21 days	0.39	0.034	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	1.33		
14 ~ 35 days	1.44	0.594	Not significant
(c) Treated Group			
-14 ~ 0 days	0.96		
21 days	0.48	0.005	Significant decrease

Table F3.6 $CD4^+$: $CD8^+$ T cell Ratio

COMPARISONS	Median	P-value	Comment
(a) Control Group			
-14 ~ 0 days	1.98		
21 days	1.09	0.005	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	1.65		
21 ~ 35 days	2.85	0.005	Significant increase
(c) Treated Group			
-14 ~ 0 days	1.73		
21 days	0.51	0.005	Significant decrease

Table F3.7 Serum IgG antibodies 35 days post infection

COMPARISONS	Median titre	P-value	Comment
(a) Control & prophylactic Groups			
Control	5		
Prophylactic	10	0.029	Significant difference
(b) Control & treated Groups			
Control	5		
Treated	0	0.029	Significant difference
(c) Prophylactic & Treated Groups			
Prophylactic	10		
Treated	0	0.029	Significant difference

F4. STATISTICAL ANALYSIS FOR PROLIFERATION AND CYTOKINE RESULTS

Table F4.1 PBMC proliferation in culture with trypanosomes

COMPARISONS	Median Stimulation Index	P-value	Comment
(a) Control Group			
-14 ~ 0 days	0.95		
21 days	0.02	0.016	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	1.37		
21 days	0.37	0.005	Significant decrease
(c) Treated Group			
-14 ~ 0 days	0.98		
14 ~ 35 days	1.15	0.037	Significant increase

Table F4.2 PBMC proliferation in culture with ConA

COMPARISONS	Median Stimulation Index	P-value	Comment
(a) Control Group			
-14 ~ 0 days	1.92		
14 ~ 35 days	1.13	<0.0001	Significant decrease
(b) Prophylactic Group			
-14 ~ 0 days	2.89		
21 days	0.33	0.0131	Significant decrease
(c) Treated Group			
-14 ~ 0 days	1.74		
14 ~ 28 days	1.07	0.0830	Not significant

Table F4.3 Sheep PBMC culture supernatant IFN-γ levels for prophylactic group

COMPARISONS	Median Absorbance	P-value	Comment
Prophylactic Group (Pre-infection)			
-14 ~ 0 days before ISMM adminstration	0.126		
14 ~ 28 days after ISMM adminstration	0.276	0.001	Significant increase
Prophylactic Group (Post-infection)			
-14 ~ 0 days before infection	0.146		
14 ~ 28 days after infection	0.146	0.435	Not significant

Table F4.4 Mice splenic cell culture supernatant IFN-γ levels

COMPARISONS	Median Absorbance	P-value	Comment
Untreated & 21days on ISMM (Cultures with LPS)			
Untreated	0.389		
21 days on ISMM	0.011	0.001	Significant decrease

Table F4.5 Mice splenic cell culture supernatant IL-12p40 levels

COMPARISONS	Median Absorbance	P-value	Comment
Untreated & 14 days on ISMM (Cultures with LPS)			
Untreated	0.142		
14 days on ISMM	0.049	0.002	Significant decrease
Untreated & 21days on ISMM (Cultures with LPS)			
Untreated	0.142		
21 days on ISMM	0.034	0.002	Significant decrease
Untreated & 21days on ISMM (Cultures with Trypanosomes)			
Untreated	0.202		
21 days on ISMM	0.020	0.002	Significant decrease

F5. STATISTICAL ANALYSIS OF BCG RESULTS

Table F5.1 *In vivo* PBMC proliferation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	20		
7 ~ 35 days	28	0.003	Significant increase
(b) ISMM			
-14 ~ 0 days	19		
7 ~ 35 days	19	0.257	Not significant

Table F5.2. Percentage of B-cell following BCG inoculation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	46		
7 ~ 21days	29	0.028	Significant decrease
-14 ~ 0 days	46		
28 ~35 days	71	0.039	Significant increase
(b) ISMM			
-14 ~ 0 days	40		
7 ~ 35 days	30	0.007	Significant decrease

Table F5.3. Percentage of $CD5^+$ T-cells following BCG inoculation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	46		
21 ~ 35 days	28	0.008	Significant decrease
(b) ISMM			
-14 ~ 0 days	46		
21 ~ 35 days	46	0.791	Not significant

Table F5.4. Percentage of $\gamma\delta^+$ T-cells following BCG inoculation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	26		
21 ~ 35 days	9	0.004	Significant decrease
(b) ISMM			
-14 ~ 0 days	23		
21 days	8	0.016	Significant decrease

Table F5.5. Percentage of $CD4^+$ T-cells following BCG inoculation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	15		
28 ~ 35 days	5	0.002	Significant decrease
(b) ISMM			
-14 ~ 0 days	16		
28 ~ 35 days	12	0.479	Not significant

Table F5.6: Percentage of $CD8^+$ T-cells following BCG inoculation

GROUP COMPARISONS	Median (%)	P-value	Comment
(a) Control			
-14 ~ 0 days	9		
7 ~ 35 days	6	0.001	Significant decrease
(b) ISMM			
-14 ~ 0 days	14		
7 ~ 35 days	9	0.084	Not significant

Table F5.7: $CD4^+$: $CD8^+$ T-cell ratio following BCG inoculation

GROUP COMPARISONS	Median	P-value	Comment
(a) Control			
-14 ~ 0 days	1.67		
7 ~ 21 days	2.40	0.002	Significant increase
(b) ISMM			
-14 ~ 0 days	1.19		
7 ~ 35 days	2.15	0.028	Significant increase

Table F5.8: Skin thickness (mm) after PPD injection 35 days post BCG

GROUP COMPARISONS	Median (mm)	P-value	Comment
(a) Control			
0 ~ 24 hrs	7.40		
48 ~ 72hrs	14.40	0.041	Significant increase
(b) ISMM			
0 ~ 24 hrs	5.70		
48 ~ 72hrs	18.50	0.093	Not significant
(c) Control & ISMM			
48 ~ 72hrs	14.40		
48 ~ 72hrs	18.50	0.818	Not significant